Individual Amino Acids – Hungry Brain and Mobile Gut

Dissertation

zur

Erlangung der naturwissenschaftlichen Doktorwürde
(Dr. sc. nat.)

vorgelegt der

Mathematischen-naturwissenschaftlichen Fakultät

der

Universität Zürich

von

Josua Hannes Jordi

von

Huttwil, BE

Promotionskomitee

Prof. Dr. F. Verrey (Co-Vorsitz)

Prof. Dr. T. A. Lutz (Co-Vorsitz)

Prof. Dr. C. Wagner

Prof. Dr. D. Tomé

Zürich, 2013

Table of Contents

1. Summary

In every living organism amino acids are pivotally important multifunctional molecules. Single amino acids drive mayor intracellular signaling pathways controlling growth and proliferation, and enable neuronal communication in the synaptic cleft. Apart from signaling, their oxidative break down in the citric acid cycle provides energy and metabolic substrates for the cell. When several single amino acids are covalently linked and correctly folded, they form a functional protein. This certainly describes one of the most important processes in biology. All these vital functions depend on the availability of amino acids. Not surprisingly, amino acid pools are maintained within a physiological range by complex regulatory feedback mechanisms – a process termed homeostasis. A homeostatic challenge is to refill the amino acid pools without perturbing amino acid homeostasis, which might cause severe side effects. The temporal regulation of transport protein expression may solve this problem in single cell organisms, whereas eating behavior has to be balanced with the homeostatic needs and the specific available nutrient source in higher species. How this is achieved is currently under active research, but little is known in the context of individual amino acids.

One reason might be, that ingested proteins are broken down to twenty different individual amino acids leading to large complexity. Not only do they differ in their chemical structure, but some are also nutritionally essential. **We hypothesize that, based on these structural differences; they may have distinct roles in the control of food intake and gastrointestinal function.** We systematically assessed the impact of all 20 individual proteogenic amino acids on food intake and gastric function. These two functional readouts are particularly relevant in the short-term regulation of nutrient intake. Food intake determines the maximal achievable intake of nutrients, and gastric function i.e. gastric emptying and secretion dictates the concentration and the timing of nutrient release into the small intestine. Both have a direct impact on plasma nutrient concentration and the timing of nutrient appearance in plasma.

Here, we show that short-term food intake was most potently reduced by oral L-arginine, L-lysine and L-glutamic acid compared to all other 17 proteogenic amino acids in the rat. As feeding behavior is controlled by neuronal circuits located in specific brain areas, we tested for neuronal activity using immunohistochemistry after L-arginine, L-lysine and L-glutamic acid application. An increased number of cFOS positive cells were detected in the blood-sensing area postrema and the nucleus of the solitary tract. To test whether circulating amino acids can directly signal to the brain to induce their anorectic effect, we administered L-arginine, L-lysine and L-glutamic acid intravenously. All three amino acids induced an anorectic response that was similar to the one induced after oral application. Surgical lesion of the area postrema abolished the anorectic responses of L-arginine and L-glutamic acid but not of L-lysine. The nucleus of the solitary tract is the main projection site of the vagus nerve which innervates the gastrointestinal tract. Surgical lesion of vagal afferents did not alter the anorectic effect of L-arginine and L-glutamic acid but of L-lysine. We presume that L-arginine and L-glutamic acid act in the area postrema to cause their anorectic response, while L-lysine stimulates hepatic vagal afferents projecting to the nucleus of the solitary tract. Interestingly, in the gastrointestinal tract all three amino acids induced gastric distension. L-arginine and L-lysine induced gastric secretion detected by changes in the alkaline tide. Gastric emptying, measured by stomach phenol red retention, was delayed after L-lysine and L-glutamic acid treatment. At the level of the small intestine, L-arginine and L-lysine accelerated phenol red dye passage into the cecum. The gastrointestinal effects of L-lysine were shown to be dose dependent in the rat and were analogously observed in healthy human subjects. The highest L-lysine dose caused self-limiting diarrhea in

humans, but no other side effects were reported. The gastrointestinal effect induced by L-arginine and L-lysine was dissociated from their effect on food intake and induced conditioned taste aversion in the rat. Hence, L-arginine, L-lysine and L-glutamic acid had a remarkable specific impact on mechanism important for food processing in the gastrointestinal tract and on food intake in rats and humans. This may suggest that they act as direct sensory input to assess dietary protein content and quality *in vivo*.

Here, we show that L-cysteine, L-lysine, L-arginine and L-tryptophan most potently delayed gastric emptying and that L-arginine and L-lysine most potently stimulated gastric secretion compared to all other proteogenic amino acids in the rat. The systematic assessment of these two key stomach functions was only feasible, because we established and validated a quantitative non-invasive high-throughput computed tomography based method. This novel method can measure simultaneously gastric emptying and secretion in rats *in vivo*. Future efforts aim to assess if gastric secretion and emptying induced by the candidate amino acids are conducted by a shared control mechanism, to identify its localization and potential effector molecules i.e. gastrointestinal hormones.

In conclusion, we revealed remarkable amino acid specificity for two critical nutritional functions, namely food intake and gastric function. The main question arising from this work is the cellular mechanism enabling the remarkable amino acid specificity. This might give insights into how individual amino acids contribute to the control of protein intake, and how protein quality is assessed at a molecular level.

2. Zusammenfassung

In jedem lebenden Organismus sind Aminosäuren wichtige multifunktionale Moleküle. Einzelne Aminosäuren regulieren wichtige intrazelluläre Signalwege, die für die Steuerung des Wachstums und die Proliferation verantwortlich sind. Ausserdem ermöglichen sie die neuronale Kommunikation im synaptischen Spalt. Neben ihrer Signalfunktion können sie durch den oxidativen Stoffwechsel im Citratzyklus in wichtige metabolische and energetische Substrate für die Zelle umgewandelt werden. Die kovalente Verknüpfung von mehreren einzelnen Aminosäuren und deren korrekte Faltung führt zur Bildung eines funktionellen Proteins. Dies sind mit Sicherheit einige der wichtigsten Prozesse in der Biologie. Alle diese lebenswichtigen Funktionen hängen von der Verfügbarkeit von Aminosäuren ab, weshalb diese in einem physiologischen Bereich durch komplexe Rückkopplungsmechanismen gehalten werden. Eine Herausforderung besteht darin, die Verfügbarkeit der Aminosäuren aufrechtzuerhalten, ohne die Homöostase des biologischen Systems stark zu erschüttern. In einzelligen Organismen kann die Regulierung der Transportproteine an der Zelloberfläche bereits dieses Problem lösen. Diese Aufgabe ist in höheren Arten komplexer, da das Essverhalten, die Verfügbarkeit von spezifischen Nährstoffen und der Bedarf koordiniert werden müssen. Wie dies erreicht wird, ist derzeit Bestandteil aktiver Forschung. Leider ist im Rahmen von Aminosäuren sehr wenig bekannt.

Ein Grund dafür ist, dass gegessene Proteine zu zwanzig verschiedenen einzelnen Aminosäuren verdaut werden, was zu einer grossen Komplexität führt. Sie unterscheiden sich nicht nur durch ihre chemische Struktur sondern auch ob sie essenziell sind. **Unsere Hypothese besagt, dass aufgrunde ihrer strukturellen Unterschiede einzelne Aminosäuren unterschiedliche Rollen für die Kontrolle der Nahrungsaufnahme und Magenfunktion haben.** Diese Hypothese wurde durch die erstmalige systematische Beurteilung der individuellen Einflüsse von Aminosäuren auf die Nahrungsaufnahme und die Magenfunktion in der Ratte getestet. Diese zwei Funktionen sind besonders in der kurzfristigen Regulation der Aminosäuren-Versorgung relevant. Die Nahrungsaufnahme bestimmt die maximal mögliche Aufnahme von Nährstoffen. Die Magenfunktion, hier die Sekretion und die Entleerung, bestimmen die Konzentration und den Zeitraum der Freisetzung von Nährstoffen in den Dünndarm und haben dadurch direkten Einfluss auf den Nährstoffgehalt im Blut und das zeitliche Erscheinungsbild nach der Aufnahme einer Mahlzeit.

In dieser Arbeit zeigen wir, dass die kurzfristige Nahrungsaufnahme in der Ratte durch oral appliziertes L-Arginin, L-Lysin und L-Glutaminsäure im Vergleich zu allen anderen 17 proteinogenen Aminosäuren am stärksten reduziert wird. Da das Fressverhalten durch Neuronen in bestimmten Hirnregionen gesteuert wird, haben wir getestet, wo L-Arginin, L-Lysin und L-Glutaminsäure neuronale Aktivität induzierten. Eine erhöhte Anzahl von cFOS positiven Zellen wurde in der *Area Postrema* und dem *Nucleus Tractus Solitarii* nachgewiesen. Die *Area Postrema* ist nicht von der Blut-Hirn-Schranke geschützt.Um zu testen, ob zirkulierende Aminosäuren das Fressverhalten beeinflussen, haben wir L-Arginin, L-Lysin und L-Glutaminsäure intravenös verabreicht. Alle drei Aminosäuren induzierten einen anorektischen Effekt, der eine ähnlich Stärke wie der nach einer oralen Applikation hatte. Eine chirurgische Läsion der *Area postrema* verhinderte den anorektischen Effekt von L-Arginin und L-Glutaminsäure, nicht aber den von L-Lysin. Der *Nucleus Tractus Solitarii* ist die primäre Projektionsregion des *Vagus* Nervs, welcher den gesamten Gastrointestinaltrakt innerviert. Eine chemische Läsion der afferenten vagalen Nervenfasern verhinderte den anorektischen Effekt von L-Lysine, nicht aber den von L-Arginin und L-Glutaminsäure. Wir gehen davon aus, dass L-Arginin und L-Glutaminsäure über die *Area Postrema* den anorektischen Effekt

verursacht, während L-Lysin diesen Effekt über vagale afferente Nervenfasern auslöst. Interessanterweise induzieren alle drei Aminosäuren eine Ausdehnung des Magens. L-Arginin und L-Lysin verursachen Magensäuresekretion, detektiert durch die Veränderung des alkalischen Gleichgewichts. Die Magenentleerung, gemessen durch die Retention von Phenolrot im Magen, ist verlangsamt durch L-Lysin und L-Glutaminsäure. Im Dünndarm beschleunigen L-Arginin and L-Lysin den Transit eines Farbstoffes in den Blinddarm. Die Wirkung von L-Lysin im Magen sowie im Dünndarm ist dosisabhängig in der Ratte, und wurde analog im Menschen beobachtet. Diese peripheren gastrointestinalen Mechanismen sind von ihrem anorektischen Effekt unabhängig und führen zu einer Geschmacksaversion. Zusammenfassend hatten L-Arginin, L-Lysin und L-Glutaminsäure eine bemerkenswerte, spezifische Auswirkung auf die wichtigen Mechanismen für die Verdauung im Magen-Darm-Trakt und auf die Nahrungsaufnahme. Dies deutet darauf hin, dass sie als direkte sensorische Signale wirken, um den totalen Proteingehalt und die Qualität der Nahrung zu messen, und daher besonders relevante Signale für das Erhalten der Aminosäure-Homöostase sind.

In dieser Arbeit zeigen wir, dass L-Cystein, L-Lysin, L-Arginin und L-Tryptophan die Magenentleerung am stärksten verzögern, und dass L-Arginin und L-Lysin die Magensäuresekretion am stärksten induzieren im Vergleich zu allen anderen proteinogenen Aminosäuren in der Ratte. Die systematische Beurteilung dieser zwei zentralen Funktionen des Magens war nur möglich, weil wir eine quantitative und nicht invasive Computertomographie-Methode entwickelt und validiert haben. Diese neue Methode erlaubt es, gleichzeitig die Magenentleerung und -sekretion in der lebenden Ratte zu messen. Zukünftige Bemühungen beabsichtigen zu entschlüsseln, ob die Magenentleerung und Magensäuresekretion,induziert durch die Aminosäuren, von einem gemeinsamen Kontrollmechanismus gesteuert werden, wo dieser Mechanismus lokalisiert ist und welche Effektormoleküle, z. B. gastrointestinale Hormone, sekretiert werden.

Zusammenfassend zeigen wir hier für zwei zentralen Funktionen in der kurzfristige Kontrolle der Nährstoffaufnahme, nämlich dem Fressverhalten und der Magenfunktion, ein bemerkenswerte Spezifität für bestimmte Aminosäuren. Die wichtigste Frage, die sich aus dieser Arbeit ergibt, ist nach den zellulären Mechanismen, die diese bemerkenswerte Aminosäuren-Spezifität ermöglichen. Die Antwort auf diese Frage ermöglicht Einblicke, wie diese einzelnen Aminosäuren auf genaueste Weise die Proteinaufnahme steuern, und wie Proteinqualität auf molekularer Stufe wahrgenommen wird.

3. Introduction

3.1 Nutrient Homeostasis

Homeostasis describes a state where a system maintains its internal variables within a relative constant range. In honor of the inventor of this concept – Claude Bernard – one often speaks of "la fixité du milieu intérieur" (156, 157). Homeostasis is a characteristic conserved in all organisms from single-cell microbes to mammals. Genes stabilizing internal variables were presumably selected because they enable other vital processes to function under stable intracellular conditions in the ever changing environment. Thereby enzymatic processes such as metabolism, gene expression, etc. can evolve towards high efficiency and are protected from the large changes in the encountered environment. The number and precision of variables regulated, and the complexity of the system regulating them increase with complexity of an organism. Single cells regulate crucial variables such as the membrane potential, pH or volume, whereas higher species in addition control the extracellular compartment (*milieu intérieur*) that contains a number of interlinked organs and cells. Impairments of these regulatory mechanisms often lead to imbalances causing disease. In sum, homeostasis enables life in the dynamic environment encountered on earth from the freezing-polar regions to the hottest Vulcan reefs.

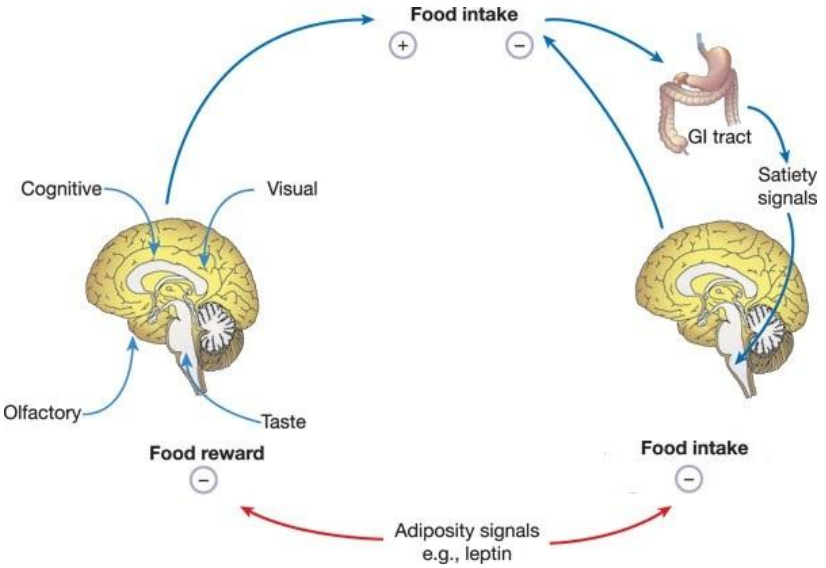


Figure 1 – Nutrient homeostasis. *The central nervous system modulates food intake to maintain stable nutrient stores. Long-term signals (red) inform about current storage size and short-term signals (blue) about immediate meal-related information. Figure was adapted from (104).*

Systemic homeostasis encounters several challenges such as the regulation of cell volume, body temperature and nutrient supply. The concept of nutrient homeostasis describes an elaborated process, which adjusts food intake over time to promote stability in the amount of stored nutrients (8, 104, 167). The central nervous system controls this balance by integrating different blood born and neuronal signals. They can be roughly separated in long- and short-term signals. The latter relays immediate signals from an on-going meal and the long-term signals mediate information related to nutrient stores (Fig. 1). For instance, leptin plasma concentrations correlate with adiposity stores, whereas CCK release with food intake. However, this separation of signals is an over simplification and functional overlaps exist as well for leptin. Importantly the current knowledge is most advanced in the field of energy intake based on its mayor implication for the current obesity epidemics. Nevertheless there are experimental indications that homeostatic control as well exists for muscle and bone mass. Next, I'll review the current understanding with a specific focus on the interaction of muscle mass and amino acid/protein intake. Amino acids are the macronutrients my research focuses on and therefore a more detailed understanding complements my thesis.

3.2 Amino Acids

Amino acids as building blocks of proteins are the most abundant component in the lean human body other than water. There are 20 proteogenic amino acids varying in size, charge, polarity and atomic composition. The main source of amino acids for metazoans is ingested protein, which are digested to individual amino acids prior to their release into portal circulation from intestinal enterocytes. The liver filters the portal blood prior entering systemic circulation and elaborated re-absorptive machinery located in the proximal tubules prevents excess excretion of amino acids into the urine (155). Depending on the specific cell types involved amino acids can either serve as signals for growth control, neurotransmission and others or simply be utilized for enzyme synthesis or as metabolic substrates. Importantly, microbe metabolism can synthesize all amino acids *de novo*, however, this trait was lost in the course of evolution and therefore certain amino acids must be ingested to support normal growth and reproduction (169). Alternatively muscle catabolism can provide vital amino acids in times of shortage. However, this autophagy process is only observed under specific conditions. Critically there is no amino acid storage site analogous to adipose tissue providing energy whenever needed. Hence, there is a homeostatic need for protein ingestion. Ingestion of proteins and nutrients in general is a regulatory challenge, because efficient absorption must be performed while maintaining amino acid respectively nutrient concentration within the homeostatic range. In the next chapters I'll review the current understanding of amino acid homeostasis starting with long-term signals regulating of amino acid intake.

3.3 Long-term Signals affecting Amino Acid Intake

The brain ultimately tunes food intake with the requirements of the body (Fig. 1). This would suggest that a difference in protein demand would stimulate specific amino acid intake. For instance, an increased protein synthesis is required to drive cell proliferation and differentiation during development. Consequently one would expect a higher protein demand in young animals compared to old. Indeed young rats consume a larger amount of high protein diets compared to mature individuals if given the choice (74, 165). Even in humans it is generally recommended to nurture young infants with additional proteins to support normal growth and development (77). In the same line of argumentation, animals with a high muscle mass due to growth hormone treatment or increased physical activity manifested an enhanced selection for protein enriched diets (29, 119, 130). Inversely, specifically restricting protein intake in animals induces an increased protein demand, and therefore a specific appetite for protein rich diets is observed following this experimental perturbation (41, 79, 166). Hence, a higher protein demand induced by different physiological perturbations stimulates increased protein intake. Importantly, these behaviors are nutrient specific as modulating energy requirements for example by cold exposure selectively increased energy intake, whereas protein consumption remained constant (84). Generally these observations indicate the presence of a signal reflecting muscle mass similar to leptin and adipose tissue. Irisin was recently suggested to potentially play such a role, but remained heavily controversial (17, 146). The identification of such a signal is a key step for a better understanding of the regulation of protein intake and as well amino acid homeostasis. Taken together, the brain balances nutrient ingestion and requirements targeting homeostasis. Consequently blood amino acid levels are relatively constant throughout the day, while eating marks the exception (169). Long-term signals were only briefly discussed, because the here presented work is not related to alterations of them. All animals used within one experiment in this dissertation had a similar age and body weight

thereby minimizing a bias due to distinct body composition. Next, I'll discuss the mechanism controlling short-term food intake, which are stronger related to the work in this thesis.

3.4 The Effect of Proteins and Amino Acids on Food Intake

Limiting food intake is key to minimize particularly large disruption of plasma nutrient homeostasis (167). The brain accomplishes not only this task through the integration of information related to the meal and its composition, but as well by taking other factors like stress, day-time, social situation or experience (learned) in to account (Fig.2). The main components of every diet are carbohydrates, fats and proteins. The "Bundesamt für Gesundheit" recommends a daily protein intake of 0.8-2.0 g/kg for a healthy adult, which reflects 10-20 % of the daily energy intake in an adult man (77). Of all three macronutrients, protein most potently delays food intake at an iso-caloric dose (92, 93). Data supporting these conclusions are abundant throughout the animal kingdom from insects, fish, rodents and humans (reviewed in (102)). Specifically a high protein diet inhibits food intake, a low protein diet increases food intake, and a very low protein diets induces a dietary rejection compared to a standard diet (7, 43, 75, 164). These findings encouraged the development of diets high in protein content (30% protein per total energy) for weight loss with the most prominent being the Atkins and Protein Power diet (reviewed in (3)). The initial concerns for a higher risk of osteoporosis, renal insufficiency or coronary artery disease were insufficient and the intake up to 35% protein per total energy is concerned as safe. Both diets stimulated significant weight loss and reduced several risk factors for cardiovascular disease in obese test persons. Particularly convincing was the observation that only fat mass was lost, but lean body mass and energy expenditure were maintained. Still these and diets in general are not successful strategies for reducing body weight in many individuals mainly due to lack of compliance with the dietary paradigm.

Unfortunately, how protein diets induce their potentially specific effect on adipose stores remained obscure. One reason might be that a diet rich in proteins is low in fats or/and in carbohydrates, thereby limiting conclusions as one or the other variable can contribute to the observed effect. Geometrical models were applied to tackle this key limitation, which enables the comparison of protein versus carbohydrates versus fat consumption in the 3D ingestive space (139). Analyses of a large number of experimental data showed, that feeding behavior can be best described in the 2D space. Therefore the energy providing macronutrients fats and carbohydrate are summed as the energy variable (65, 140). Interestingly, this approach indicates that several species regulate ingestive behavior around a specific protein:energy target (124). This concept was recently experimentally validated in mice and humans (59, 68, 94, 142). Interestingly it was even demonstrated that insects and rodents prioritize protein over energy intake, if they cannot reach their protein:energy target. This led to the formulation of the "obesity leverage" theory, which proposes that the decreased protein content relative to energy in the western diet provokes a substantial increase in energy intake causing the current obesity epidemic (140). However, a certain controversy exists as certain studies do not support an anorectic effect of a high protein diet in humans (13, 92, 93, 123). These differences might be due to the fact that not only total dietary protein quantity but as well quality differentially impacts on food intake. When protein quality was altered, differences in their anorectic effect were observed in rodents and humans (19). Exemplary in rats soy proteins do not alter food intake in the same magnitude as yeast proteins (44). As different protein sources have a different amino acid composition, these studies might indicate a role for individual amino acids in the control of food intake (6, 44, 121). In agreement, rats freely select from two diets, each alone deficient but together complementary in their amino acid profile, to meet balanced amino acids intake (49).

Critically these studies as well exclude the possibility that animals only consume proteins to fuel their nitrogen needs (79, 82, 100). The impact of individual amino acids on food intake was not studied systematically in the past. Of all the 20 proteogenic amino acids only 12 where tested and this under different experimental conditions thereby disabling comparison (4, 5, 12, 107). Hence, their individual significance remains unclear. In summary, protein quantity and quality has a profound impact on feeding behavior, whereas currently the precise role of individual amino acids for satiation remains unknown.

The importance of individual essential amino acids was recognized already early based on the impact of an extreme dietary paradigm. If a diet is depleted completely of an individual essential amino acid, animals reject this diet within 20 min (reviewed (57)). When the missing amino acid is provided within another diet, animals consume the right proportion of both to reach their protein requirements (49). Alternatively this rapid rejection can be reversed by injecting the limiting amino acid into the anterior piriform cortex or infused into the carotid artery, whereas the injection into the jugular vein or application of the non-limiting amino acid has no effect (86, 131). This deficiency of a single essential amino acid is sensed by the accumulation of uncharged tRNA's and activation of the highly conserved GCN2 pathway in the anterior piriform cortex (62). Lesion of the anterior piriform abolishes this selective rejection of a deficient diet, but does not alter the anorectic effect of high protein diets (85). Hence, there is a clear dissociation of the response to insufficiency and physiological variations in protein quantity therefore the importance of these findings for physiological conditions might be limited.

Provocatively one can question whether the behavioral responses to proteins described above represent a specific effect to protein intake or alternatively whether they are a secondary effect to the macronutrient. The intake of a high protein diet might lead to toxic levels of amino acids in the blood stream and therefore provoke a protective mechanism leading to an unspecific avoidance of an unhealthy diet. Vomiting or diarrhea reflect such an extreme physiological reactions to maintain or protect homeostasis either from nutrients or toxic elements in the diet. To test this hypothesis, conditional taste aversion tests were conducted in which rats were exposed to diets very high in protein content (>60%) (10, 87). However, animals failed to avoid these high protein diets. Quite the contrary was actually observed. A conditioned learned preference was elicited, when proteins or amino acids were infused directly into the gut (116, 117). This holds as well for sugar or fat infusion therefore potentially reflecting a general response to a gastrointestinal nutrient load. Furthermore if animals are allowed to self-select between diets different in dietary protein content, they do not show a preference for low or high proteins diets, but select randomly to meet their protein requirements. These data collectively suggest that there is a specific mechanism to regulate protein intake in terms of quantity and quality intake. In the next chapter I'll discuss the current known signals induced by a protein meal.

3.5 The Short-Term Control of Protein and Amino Acid Intake

The brain integrates a large variety of signals to ultimately conduct nutrient intake within a homeostatic range. Meal born signals are extremely diverse, redundant and differentially induced by macronutrients (Fig. 2). They differ in the temporal occurrence, their origin and their reliability to predict the macronutrient or energy content of a meal. In the next few sections I'll review the different signals following the anatomical travel route of a meal. This is not a statement of importance or priority but rather an approach to simplify the structure. Critically all these signals are

induced in a narrow time window and consequently may overlap, which might provoke synergies or neutralization.

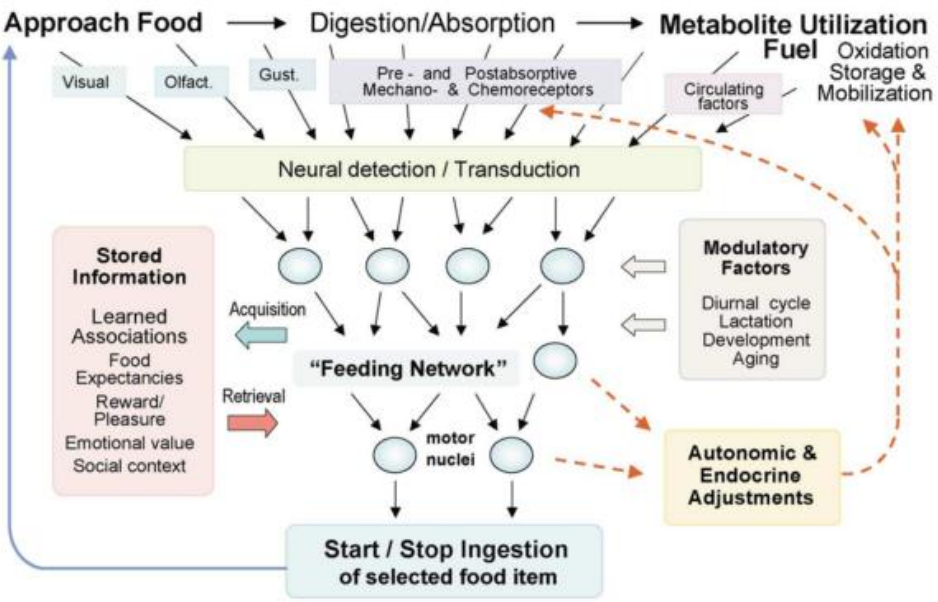


Figure 2 – General information flow contributing to the neuronal control of food intake. *Important is the circulatory nature of the diagram illustrating that a sequence of ingestion may be initialized or terminated by any of the nodes in diagram. The figure was adapted from (11).*

Taste, Smell, Sight and Texture

Taste, smell, sight and texture of a meal are obvious signals related to food and might help to predict the protein content of a diet directly or based on learned association. Indeed the taste of several amino acids, termed umami, is tasty for animals and humans. The heterodimer G-protein coupled receptor T1R1/T1R3 mediates the umami taste in mammals and is expressed in taste cells distributed randomly over the tongue. This amino acid taste receptor is broadly tuned and responds not only to umami (Monosodiumglutamate) but also to most other 20 proteogenic amino acids (106). Excitingly the cell type solely determines the behavioral response (preference or rejection), whereas the taste receptor the selectivity to the taste (172). Hence, membrane transport or intracellular metabolism does not play a role for taste detection. However, T1R1/T1R3 knockout mice have a diminished but not an abolished behavioral and neuronal response to umami compounds indicating other forms of amino acid taste detection (38). Interestingly T1R1/T1R3 and other taste receptors are not only expressed in the oral cavity, but as well in the gastrointestinal tract among others. Recently it was shown that the intestinal sugar transport by SGLT-1 and CCK release is controlled by T1R1/T1R3 in the small intestine suggesting a broader physiological role for taste receptors (37). No data is currently available of the eating behavior of T1R1/T1R3-knockout mice exposed to a high protein diet. Nevertheless, altering dietary taste or texture did not prevent the anorectic effect of a high protein diet in rats suggesting post-oral mechanism in control of this behavior (88). This might not be surprising as most proteins are still folded and located in their respective cellular compartment, thereby hidden from detection by tongue receptors. Obviously this only holds for uncooked food. In summary, taste, smell, sight and texture are essential for the binary decision – to eat or not to eat, whereas their importance to modulate specific protein intake might be limited. That said the underlying taste receptors might be a promising mechanism for nutrient detection, if expressed at a different localization. The broad amino acid specificity of the T1R1/T1R3 receptor, however, might be problematic to explain the behavioral responds to alterations in protein quality discussed above.

Stomach

The stomach is a reservoir for ingested food and plays a key role for the primary digestion of nutrients. Gastric acid secretion mediates the denaturation of proteins and activates different digestive enzymes among them pepsin. Pepsin cleaves proteins into di/tri-peptide or single amino acids. This digestive process is continued in the duodenum by trypsin/chymotrypsin, which are secreted by the pancreas (157). Stomach function and its regulation will be discussed in a subsequent section with more detail; here the focus is given to its potential role within the context of food intake regulation. Based on the nutrient digestion the stomach is the primary location where the body is exposed to single amino acids and therefore potentially the primary place for their detection. However, evidences supporting this are still lacking. Nevertheless mechano-sensitive receptors in the stomach where shown to relay neuronal signals via vagal or splanchnic afferent fibers to the hindbrain and other brain areas. These receptors were shown to be stretch and tension sensitive, hence, signaling gastric distension (137, 167). Obviously, gastric distension was then hypothesized to play a role in food intake regulation (reviewed in (73)). Pyloric cuff experiments in rats supported this hypothesis and gastric balloon inflation studies showed a significant correlation between inflation and perception of fullness, satiation and gastric discomfort in humans (40, 48, 64, 114, 158). If feeding behavior was assessed within balloon inflation studies, no effect was observed in humans (114). In rats only extensive gastric distension leads to food intake reduction, whereas small distension up to 4 ml had no effect (71). Therefore physiological gastric distension might play a role for the perception, but seems not to control food intake directly. Additionally it remains unclear, how a chemical signal (the amino acid profile of a meal) should be translated solely by mechanical distension. In summary the stomach enables chemical, enzymatic and mechanic digestion of food and relays mechanical stimuli to the brain.

Small Intestine

The small intestine is the primary site of nutrient absorption. Individual amino acids are absorbed by a large family of different transport proteins located at the apical membrane of enterocytes. Alternatively, di/tri-peptides are absorbed via PepT1 transporter and subsequently catabolized to individual amino acids. Importantly only individual amino acids are released into the portal blood by basolateral amino acid transporters. Hence, individual amino acids in the blood stream could elicit the behavioral response to a high protein diet as their concentration in circulation directly correlates with the amount of ingested proteins (118). Apart from enterocytes as well endocrine cells are located in the small intestine, which secret a large variety of gastrointestinal hormones involved in food intake regulation. STC1 cells, an *in vitro* model for intestinal endocrine cells, increase intracellular [Ca^{2+}] upon stimulation with specific amino acids. [Ca^{2+}] facilitates vesicle fusion to the plasma membrane, a mechanism common for neurotransmitter and hormone release (32, 170). Indeed, the release of the gut hormone GLP-1 from primary murine intestinal cells correlated with intracellular [Ca^{2+}] fluctuation induced by specific individual amino acids (147). Similar amino acid specific findings were reported for the release of CCK (159). Hence, this demonstrates a remarkable amino acid specificity of the secretory function of endocrine cells *ex vivo*. *In vivo* concentrations of CCK, GLP-1, amylin, grehlin, GIP, PYY, glucagon and insulin were altered after a high protein stimulus, but conflicting findings were as well reported (19, 20, 55, 83, 101, 113, 125, 141). Interestingly PYY knockout mice are selectively resistant to the satiation effect of a high protein diet indicating a specific role for PYY (9). However, these results have to be interpreted conservatively as the phenotype of PYY knockout mice varies largely between different labs under normal feeding

conditions (16, 134). Finally if one considers that fat and carbohydrates as well modulate the secretion of gastrointestinal hormones, it remains unclear how gastrointestinal hormones should mediate the macronutrient specific ingestive behavior described for proteins. Taken together the small intestine enables amino acid absorption into portal circulation and the secretion of a large variety of gut hormones.

Hepatic Portal Vein

The blood from the small intestine flows to the liver through the hepatic portal vein. Importantly, this blood is on the first passage still unfiltered by the liver and therefore thought to be particularly rich in nutrients and gastrointestinal hormones acutely after a meal. The blood gets diluted only after entering general circulation by the hepatic veins. Exceptions are the branch chain amino acids (L-leucine, L-isoleucine, L-valine) which are thought not to be altered by liver metabolism (169). Importantly, blood amino acid levels change within 10 min after the intake of a meal and show a dose dependent increase correlating with the amount of ingested proteins (118). The rise in amino acid concentration in the blood plateaus within 30 min after ingestion. By the blood stream, changes in amino acid concentration reach all organs including the brain. Hence, acute amino acid changes in the blood stream correlate with protein intake.

Vagus Nerve

The vagus nerve links the gastrointestinal tract to the brain projecting mainly to the nucleus of the solitary tract. The vagus nerve splits after the diaphragm into three branches – the hepatic, cephalic and gastric (112). Importantly vagal dendritic ends reach close to the absorptive enterocytes in the small intestine, but not into the gastrointestinal lumen. Hence, nutrients must be absorbed for direct vagal activation or mediate their effect indirectly by a paracrine mechanism. 80 % of vagal fibers are afferents and thought to serve as mechano- or chemo-sensors. Mechanical stimulation of the stomach wall by intragastric balloon inflation stimulates gastric vagal firing *in vivo* and induces cFOS expression in the nucleus of the solitary tract (48, 137). Several *ex vivo* electrophysiological studies revealed the amino acid specificity of vagal firing as different amino acids excite or inhibit vagal afferent activity dependent on the exposed vagal branch (109–111, 145, 151). Interestingly of all amino acids tested only intragastric L-glutamic acid induces vagal signaling *ex vivo* (78, 154). Not surprisingly a duodenal milk protein infusion as well induces vagal afferent firing showing the importance of vagal firing under more physiological conditions (149). Additionally to nutrients, several gastrointestinal hormones mediate their anorectic effect by vagal afferents. CCK, GLP-1, PYY and grehlin do not alter feeding behavior in animals with a lessioned vagus nerve (1, 52). To the contrary a high protein diets still reduces food intake in total vagotomized or capsaicin treated (lesion specific for vagal afferents) rats (89, 148). Hence, high protein diets induce vagal signals and the release of gastrointestinal hormones, but their relevance to regulate specific protein intake is not convincing. Their role might be to specifically adjust gastrointestinal digestive function (motility, secretion, pancreatic function, …) to the macronutrient composition of the ingested meal.

Central Nervous System

The central nervous system ultimately controls eating behavior. The large variety of cells, their connectivity and the high-temporal resolution of neuronal activity make the brain one of the most formidable and complex system to understand. Numerous neurons form anatomically defined brain areas which presumably are relevant for overlapping functions. Efforts to generate a functional brain map were conducted, but remain difficult to understand due to the extensive connectivity and

plasticity (11). In context of feeding behavior the hypothalamus received a large amount of attention presumably because the homeostatic long- and short-term signals interact at this brain region (Fig. 1). Nevertheless the brain stem is of broader interest for short term signals, because the vagus nerve primarily projects to the nucleus of the solitary tract and the area postrema is thought to sense blood born signals. The most simple and direct mechanism to detect protein intake would be neurons sensing amino acids. Blood amino acid concentrations correlated with the amount of protein ingestion and with total brain amino acid concentrations (118). Even more elegant micro-perfusion studies revealed feeding dependent increases in brain amino acid concentrations in freely moving animals in different brain regions (25–28). This demonstrates that dietary and therefore circulatory amino acids have access to the brain in a reasonable time to contribute to the control of food intake. Alternatively amino acids could be detected within one of the circumventricular organs, which are not protected by the blood brain barrier for instance in the area postrema or the subfornical organ to mention two. Evidence that neurons can directly sense nutrients and thereby modulate food intake were first described for glucose and lipids, newer reports suggesting similar mechanisms for amino acids (34). The amino acid L-leucine was shown to modulate neuronal activity of hypothalamic cells by an mTOR (mammalian target of rapamycine) dependent mechanism (35). Similar mTOR dependent L-leucine detection was recently described in the nucleus of the solitary tract located in the brain stem (15). mTOR activation in both studies lead to a decrease in food intake. However, the role of this effect was challenged by the observation that physiological changes in plasma L-leucine concentration by dietary supplementation or genetically induced did not inhibit food intake (81, 102, 105, 122, 171). Alternatively mTOR independent mechanisms for amino acid sensing by neurons were as well described. Hypothalamic orexin/hypocretin neurons were electrically more potently excited by non-essential amino acids than essential (76). The authors suggested a dual mechanism for detection involving the inhibition of K_{ATP}-channels and the activations of system-A amino acid transporters. Taken together, dietary amino acids have direct and quick access to the brain and thereby may stimulate amino acid sensitive neurons. The physiological importance of this afferent route and their overlap with peripheral signals remains to be disentangled.

In sum, throughout animal kingdoms a protein diet inhibits food intake more potently than carbohydrates or fats under isocaloric condition. Even though proteins can induce peripheral neuronal and endocrine mechanisms, there is no convincing data supporting this as the primary afferent pathways responsible for the anorectic effect. Currently evolving is the concept of direct amino acid detection in the central nervous system thereby enabling amino acid specific eating behavior presumably to detect protein quality. Most likely all signals can inhibit food intake, if they are experimentally specifically triggered and thereby empowered out of the temporal or physiological context. In reality, all of them might help to fine tune behavioral or other still unknown functions presumably inducing a selective advantage. If not evolution would have selected against them and their trait would have been lost.

3.6 Gastric Function

The stomach and its function receive in this thesis a larger attention, because a part of my project was devoted to the development of methods to measure its functions. The stomach is located between the esophagus and the small intestine and plays several important roles for nutrient digestion with its secretory, endocrine and motor functions (157). Nevertheless it must be noted, that the stomach is not required for survival. For instance total gastrectomy patients, providing a adapted diet, maintain adequate health and longevity (157). Less extensive but still substantial

reduction of stomach size within one of the diverse gastric bypass surgeries does neither lead to mayor absorption problems (8, 42). Anatomically the stomach can be roughly divided into three parts: First the cardia is a region distal to the gastroesophageal sphincter, the fundus is the largest part of the stomach and the distal antrum separates the stomach from the small intestine by the pyloric sphincter. The most relevant functional aspects of the stomach can roughly be divided into secretory and motor functions. Both will be discussed in the next section.

Gastric Secretion

Gastric secretion affects the kinetics of digestion and absorption. The acid environment in the stomach provokes protein unfolding and leads to the autocatalytic activation of different gastric proteases. Furthermore the extent of secretion defines the concentration of nutrients and thereby indirectly contributes to nutrient absorption in the small intestine (23). Beyond digestion gastric secretion is critical to protect the upper gastrointestinal tract from bacterial colonization and infections. For instance patients with decreased gastric secretion are more prone to enteric infections by *H. pylori*, *E. coli* or *V. cholera* (22, 97). Gastric secretion is mediated mainly by two cell types - the chief cells, which secreted pepsinogens but not acid, and the parietal cells mediating acid secretion (135, 136, 157). Upon stimulation of the later, vesicle containing H,K-ATPase and K^+ and Cl^- channels fuse to the canalicular membrane. H^+ extrusion drives gastric secretion in exchange for K^+, which is recycled to the stomach lumen by the K^+ channels. Cl^- moves passively into the stomach lumen. To energize the process parietal cells have a large amount of mitochondria performing oxidative phosphorylation and the Na,K-ATPase at the basolateral membrane uses ATP units to maintain/generate the electrochemical Na^+, K^+ gradient. Importantly the H,K-ATPase can be inhibited either directly using omeprazole or indirectly by inhibiting the Na,K-ATPase with ouabain. Both inhibitors are specific and do not interfere with each other. Stimulation of parietal cells can be direct or indirect via enterochromaffin-like cells (ECL) located in the lamina propria. The pathway by which a stimulus acts can be disentangled by inhibiting Histamine-2-receptors (cimetidine or ranitidine) as Histamine is the primary neurotransmitter released from ECL cells. Nevertheless parietal cells as well express a large number of receptors and therefore can be stimulated directly for instance by acetylcholine released from the enteric nervous system or different hormones such as CCK or gastrin. The most prominent inhibitor of gastric secretion is somatostatin. The number of hormones affecting gastric secretion is still increasing, however, for most it is still unclear if they can act as well by the enterogastric route and not only if administered intraperitonal. Most probably there is a large redundancy within these pathways thereby guaranteeing robustness of this digestive mechanism.

Gastric Motor Function

Gastric motor activity serves three functions (23, 157). First, swallowed food has to be accommodated thereby the stomach serves as a nutritional reservoir. This is achieved by a neurally mediated reflex inducing a relaxation in the fundus with no change in intragastric pressure. Second, the ingested food has to be churned and digested – this is particularly true for solids. Solids move in a process termed propulsion from the fundus to the antrum following peristaltic waves, which are initiated by gastric pacemakers located in the great curvature. If solids did not reach a size smaller than 2 mm in humans, they cannot pass the pylorus and are trapped in the antrum. There the solids are grinded by arriving peristaltic waves and moved backward following the trough of the peristaltic wave, a process termed retropulsion. Third, the pylorus and the small intestine determine the rate of gastric emptying. Solids empty similar to liquids, but describe a two phase process. An initial lag phase required for churning and grinding followed by a general emptying phase which is similar

between solids and liquids. Therefore several research teams focus on the effect of liquid emptying and do not assess solid emptying additionally. Gastric emptying is modulated by diverse signals, which are discussed in the next section.

Regulation of Gastric Function

Even though here gastric emptying and secretion were individually discussed both interact and synergize during digestion. Here, I aim to give a better integrated view of both processes upon meal ingestion and a focus is given to the impact of proteins and amino acids. The responses are complex and are historically separated into 3 phases: cephalic, gastric, and intestinal. Obviously this is a necessary simplification and considerable overlap exists.

The cephalic phase is the initial phase, where different gustatory, olfactory and visual stimuli "prepare" the stomach for the ingested food. These sensory inputs are mediated solely by the vagus nerve and are thought to mediate up to >30-40 % of the overall secretory response to a meal, whereas the impact on motor function is less well understood (23).

The gastric phase includes all mechanism induced within the gastric tract. The most critical function is the extent of gastric emptying. Non-nutrient liquids empty exponentially, whereas a more linear emptying is observed with increasing nutrient and caloric content of a meal. The rate of gastric emptying is primary determined by the caloric content of the ingested meal. It is generally accepted that a human stomach empties at a rate of ~200 kcal/h, whereas other meal properties such as volume, osmolality or macronutrient composition have a minor effect (70, 96). However, this view has been challenged by reports showing that high protein diets delay gastric emptying in an isocaloric setup in rats and humans (13, 44). Controversially other reports show no effect of a high protein load (58). Hence, the chemospecificity of gastric emptying remains debatable. Gastric secretion is known to be strongly induced by gastric distension and high protein diets (157). L-phenylalanine can induce gastric secretion indicating an even more amino acid specific effect (95). The most important hormone induced during the gastric phase of a meal is gastrin (136). Gastrin is responsible for at least 50 % the meal induced gastric secretion and mediates its effect directly via CCK_2 receptors on parietal cells and indirectly by stimulating histamine release from ECL cells. Gastrin was shown to be released upon stomach distension, but whether its release can be differentially induced by meals macronutrient content remains unclear. The importance of the gastric phase for the intestinal absorption is highlighted by a study, which showed that gastric emptying determines to >30% of the variance observed after an oral glucose load (67). This potentially as well accounts for the strong variations observed after oral glucose tolerance tests (72).

During the intestinal phase nutrients are absorbed by enterocytes, they can also stimulate the release of several hormones from endocrine cells along the intestinal tract and they can mediate a vagovagal reflex to modulate gastric emptying and secretion. Most prominently it was shown that the glucose infusion into the duodenum calibrates the gastric emptying rate so that ~200 kcal/h empty (21). However, it remains unclear to which extent portal glucose stimulates vagal afferents or if the release of insulin, GIP, CCK, GLP-1 or other gastrointestinal hormones contribute to this precise gastric emptying mechanism. Nevertheless it is clear that a protein rich meal *in vivo* or individual amino acids *in vitro* can stimulate specific hormone release as discussed above. Indeed it was shown that in rats proteins delay gastric emptying and that this effect can be antagonized by application of devazepide (CCK-antagonist) (126). Importantly, the inhibition was not complete suggesting other non-CCK mediated mechanism. Hence, GI hormones do play a role in adjusting gastric emptying and secretion. As they can be stimulated by different macronutrients (fats, carbohydrates and proteins),

there are considerable indications that gastric emptying is controlled by more than the caloric equivalent of a meal. Additionally it remains to be deciphered how these different endocrinel, paracrinel, neuronal and meal related signals interact to control gastrointestinal function.

In summary, the stomach plays an important role for the accommodation, digestion and the release of nutrients into the small intestine. Gastric emptying and secretion are the main modulatory functions and their regulation integrates neuronal, paracrine, endocrine and meal-related signals.

3.7 Aim of this Study

Essential for the understanding of this thesis is the knowledge that the side chains of amino acids are diverse leading to specific chemical characteristics. In fact, there are 20 proteogenic amino acids varying in size, charge, polarity and atomic composition. **We hypothesize that based on these structural differences they may have distinct roles in the control of food intake and gastric function.**

Our overall strategy is to systematically test the impact of all proteogenic individual amino acids on food intake and on gastric function, compare their effect strength and decipher the mechanism of the most potent modulators.

Aim 1 – Which amino acid most potently modulates food intake?

We systematically tested the impact of all proteogenic amino acids on food intake and deciphered their neuronal afferent pathway. Additionally we showed that they distinctly alter gastrointestinal function and that the gastric and behavioral effects are dissociated. This data is summarized in the manuscript entitled: “Specific Amino Acids Inhibit Food Intake via the Area Postrema or Vagal Afferents.”.

Aim 2 – Which amino acid most potently modulates gastric function?

We aimed to systematically test the impact of all proteogenic amino acids on gastric function. Here, we encountered a technological hurdle as no current method can assess simultaneously gastric secretion and emptying *in vivo* (56, 144). Additionally current methods are lethal, invasive or technically limited and therefor disable systematic studies. To overcome this limitation, we developed a novel method based on computed tomography to assess these two functions. The method was extensively tested and validated leading to a manuscript entitled: “Simultaneous assessment of gastric emptying and secretion in rats by a novel computed tomography based method”. This method provided the basis to address the above question. We tested the impact of all proteogenic amino acids on gastric emptying and secretion *in vivo*. This data is shown in the unpublished result section of this thesis.

Aim 3 – Can the effect of L-lysine be translated to humans?

Within the systematic study “Specific Amino Acids Inhibit Food Intake via the Area Postrema or Vagal Afferents.” the amino acid L-lysine showed a remarkable effect on food intake, gastric emptying and secretion. We questioned whether these effects can be translated to humans. Therefore we conducted a dose depend study of the effect of L-lysine on food intake in rats and on visual analog scales in humans. Additionally we assessed gastrointestinal function in rats based on lethal methodology and magnetic resonance imaging in humans. The findings of this translational study are subject to publication in a manuscript entitled: “L-Lysine dose dependently delays gastric emptying and increases intestinal fluid volume in humans and rats”.

Overall we showed a remarkable amino acid specific impact on several important nutritional functions. The underlying mechanism was deciphered within the physiological context. Still open remains the question for the cellular mechanism of specific amino acid detection at a molecular level.

4. Original Research Article: “Specific Amino Acids Inhibit Food Intake via the Area Postrema or Vagal Afferents.”

This section contains an original research article that was published in the Journal of Physiology on July 29, 2013.

My contribution to this paper includes the study design and data interpretation under the supervision of F. Verrey and T. Lutz. I performed all experiments including the surgeries and wrote the paper with the help of F. Verrey and T. Lutz. Additionally I supported S. Camargo writing the ZHIP grant prolongation.

Title: Specific amino acids inhibit food intake via the area postrema or vagal afferents.

Authors: Josua Jordi[1,2,3], Brigitte Herzog[1,3], Simone M. R. Camargo[1,3], Christina N. Boyle[2,3], Thomas A. Lutz[2,3,*], François Verrey[1,3,*]

Affiliations: [1]Institute of Physiology, University of Zurich, Switzerland; [2]Institute of Veterinary Physiology, University of Zurich, Switzerland; [3]Zurich Center of Integrative Human Physiology, University of Zurich, Switzerland;[*]These authors contributed equally to this work.

Additional Information:

A) Running Title: Most anorectic amino acids

B) Keywords: amino acids, food intake, gastrointestinal function

C) Total words in the paper excluding references and figure legends: 4418

D) Corresponding Author: François Verrey, Prof. Dr. med.
Institute of Physiology, University of Zürich
Winterthurerstr. 190, CH-8057 Zürich, Switzerland
Phone +41 44 635 50 44/46 Fax +41 635 68 14
verrey@access.uzh.ch

E) Table of Contents: Integrative

Key Point Summary:

- Proteins are more satiating than fats or lipids. Proteins are built by the 20 proteogenic amino acids.
- Here, we identified L-arginine, L-lysine and L-glutamic acid as the most potent anorectic amino acids in rats.
- L-arginine and L-glutamic acid require intact neurons in the area postrema to inhibit food intake, whereas L-lysine intact afferents fibers of the vagus nerve. All three mediate their effect by the blood stream.
- All three amino acids induce gastric distension by delaying gastric emptying and inducing secretion. However, the gastric phenotype does not mediate the anorectic response.
- These results unravel amino acid-specific mechanisms regulating digestion and eating behavior and thereby contribute to the understanding of nutrient sensing *in vivo*.

Word count: 113

Abstract:

To maintain nutrient homeostasis the central nervous system integrates signals that promote or inhibit eating. The supply of vital amino acids is tuned by adjusting food intake according to its dietary protein content. We hypothesized that this effect is based on the sensing of individual amino acids as a signal to control food intake. Here, we show that food intake was most potently reduced by oral Arg, Lys and Glu compared to all other 17 proteogenic amino acids in rats. These three amino acids induced neuronal activity in the blood-sensing area postrema and the nucleus of the solitary tract. Surgical lesion of the area postrema abolished the anorectic response to Arg and Glu, whereas vagal afferent lesion prevented the response to Lys. These three amino acids also provoked gastric distension by differentially altering gastric secretion and/or emptying. Importantly, these peripheral mechanical vagal stimuli were dissociated from the amino acids’ effect on food intake. Thus, Arg, Lys and Glu had a selective impact on food processing and intake suggesting them as direct sensory input to assess dietary protein content and quality *in vivo*. Overall, this study reveals novel amino acid specific mechanisms for the control of food intake and of gastrointestinal function.

Abbreviation List:

4V, 4th ventricle; Ala, L-alanine; AP, area postrema; Arg, L-arginine; CCK, Cholecystokinin; DMX, dorsal motor nucleus vagus nerve; GLP1, Glucagon-like Peptide 1; Glu, L-glutamic Acid; GR, gracile nucleus; HK, high L-lysine dose (6.7 mmol/kg Lys); IG, intragastric; IP, intraperitonal; IV, intravenous; Leu, L-leucine; LK, low L-lysine dose (2 mmol/kg Lys); Lys, L-lysine; mTOR, mamaliane target of rapamycin; NTS, nucleus of the solitary tract; Phe, L-phenylalanine; PYY, Peptide YY; RLA, ringer lactate; Trp, L-tryptophane; Tyr, L-tyrosine.

Introduction:

Survival of an organism depends on its capability to adjust eating to its nutritional requirements. The brain ultimately controls this balance by integrating diverse signals related to the nutritional status of the body and the ingested meal both in terms of its energy- and macronutrient content (Morton et al. 2006). Meal born signals can be sensed centrally in specialized brain areas or be relayed from the periphery by abdominal vagal afferents, all finally leading to meal termination. The induction of these satiating signals might be triggered directly by circulating nutrients, indirectly by hormones released from enteroendocrine cells or via mechanical stimuli such as gastric distension as discussed in several reviews (Morton et al. 2006; Woods, 2009; Fromentin et al. 2012). In many cases, these mechanisms were studied for their role in energy homeostasis, whereas their macronutrient specificity was subject to less extensive research.

Protein intake is a necessity to supply the organism with essential amino acids and species as diverse as insects, fish, rodents, and humans adjust food intake according to its dietary protein content (Morrison et al. 2012; Anderson et al. 2004). The sensory mechanisms eliciting this behavior are however debated. Taste, smell, sight and texture of a meal have been suggested to help identifying its protein content based on learned associations, but experimental alterations of these cues did not prevent the anorectic effect of a high protein diet in rodents (L'Heureux-Bouron et al. 2004). Physiological sensory mechanism detecting the protein content of a meal might therefore originate from the gut or be post-absorptive for instance in peripheral nerves or directly in the brain. Indeed, the digestion of proteins to amino acids in the gastrointestinal tract stimulates vagal and endocrinal signals including CCK, GLP-1 and PYY release (Tome et al. 2009; Blom et al. 2006). However, total vagotomy

did not interfere with the anorectic effect of a high protein diet (L'Heureux-Bouron et al. 2003) and the specificity of gastrointestinal hormones for protein intake is debatable as lipids and carbohydrates induce similar hormonal responses (Morrison et al. 2012). An alternative possibility is that amino acids may act directly on nutrient sensing neurons. Indeed, it was shown that the injection of the amino acid Leucine directly into the brain ventricles inhibits food intake via modulation of mTOR-signaling (Cota et al. 2006; Blouet & Schwartz, 2012; Morrison et al. 2007). However, the role of this effect was challenged by the observation that physiological changes in plasma Leu concentration did not inhibit food intake even though circulating amino acids have access to the brain (Laviano et al. 2006; Zhang et al. 2007; Purpera et al. 2012; Nassl et al. 2011; Potier et al. 2009; Morrison et al. 2012). Importantly, all other 19 individual amino acids were so far not studied. Hence, the sensory mechanisms tuning ingestion of food to the dietary protein content remains elusive.

Here, we hypothesize that specific individual amino acids act as a physiological signal to control food intake. First, we systematically investigated which individual amino acid given by oral gavage had a specific impact on food intake in rats. We showed that Arg, Lys, and Glu were significantly more active than all other proteogenic amino acids at an isomolar dose. To localize their respective sensory mechanism, we performed surgical invalidations that demonstrated in the case of Arg and Glu the involvement of area postrema neurons, and in the case of Lys of vagal afferents, respectively. Interestingly, in the gut all three amino acids induced gastric distension by specifically inducing secretion or delaying gastric emptying. However, these peripheral mechanical stimuli were not linked to the control of food intake as intravenously applied Arg and Lys inhibited food intake, but did not modulate gastrointestinal function. These results demonstrate for the first time the importance of Arg,

Lys and Glu for the independent adaptation of digestive functions and eating behavior probably as a direct sensory input to evaluate dietary protein content and quality.

Methods:

Ethical Approval

All procedures for rat handling and experimental interventions were according to the Swiss Animal Welfare laws,approved by the Kantonale Veterinäramt Zürich and conform to the principles of UK regulations.

Animal care

Male Wistar rats (Janvier, France) were individually housed in wire-mesh hanging cages (room temperature 21 ± 1° C, artificial 12/12 h light dark cycle, water ad libitum, rat chow-3430 Kliba Nafag, Kaiseraugst, Switzerland).

Application and euthanasia

Intragastrically administered L-amino acids (freshly prepared, Sigma-Aldrich, Buchs, Switzerland) were applied in 2 ml tap-water. 6.7 mmol/kg was selected as a single dose for the screen based on comparison to other previous publications (Ng and Anderson, 1992; Bialik et al. 1989). The plasma amino acid changes measured after individual amino acid administration (S. Tab. 2) are in the range observed after the intake of a high protein diet (Peters & Harper, 1987).Intravenously administered L-amino acids were dissolved in Ringer-lactate and adjusted to pH 7.2. Animals were euthanized using pentobarbital (IP, 100 mg/kg, Kantonsapotheke Zürich, Switzerland) in combination with isoflurane (5 %) for quicker induction.

Measurement of food and water intake

Food/water intake was measured manually by weighing food containers/water bottles or using an automated system (BioDAQ, Research Diets, New Brunswick, USA). A meal was

defined by the intake of at least 0.25 g chow, and the intermeal interval criterion was set to 10 min.

Blood analysis

Blood was collected into EDTA-coated tubes (Sarsted, Sevelen, Switzerland), inverted and centrifuged (1000 g, 10 min, 4 °C). Plasma amino acid concentration were measured by the Functional Genomics Center Zurich using high pressure liquid chromatography, plasma ions by enzymatic detection with Picollo Renal Functional Panel (Abaxis, Darmstadt, Germany) and plasma glucose by AccuCheck Aviva (Roche, Basel, Switzerland). Gastric secretion was previously shown to correlate with base excess in blood, an effect termed alkaline tide (S J Rune, 1966; Niv et al. 1993).

Phenol red quantification

Phenol red (Sigma) was quantified as described by others (Tsurugizawa et al. 2009). Phenol red recovery was 57.5 ± 0.6 % (Fig. S3A), respectively 74.3 ± 0.9 % (Fig. S3B) of the administered oral dose with no significant differences between treatment groups, thereby enabling comparison.

cFOS immunohistochemistry

This time point after gavage was chosen, because cFOS expression is strongest 90-120 min post-stimulation(Watts et al. 2006). Animals were anesthetized with pentobarbital (no isoflurane) and perfused transcardially with ice-cold phosphate buffer (0.1 M PB), followed by 4 % paraformaldehyde in PB. After removal brains were kept in paraformaldehyde for 2 h to achieve proper tissue fixation. Following incubation in 20 % sucrose solution (in PB, 48 h, 4°C) brains were snap frozen in hexane. Coronal sections (20 µm) were cut in a cryostat (CM 3050 Leica, Germany) throughout the brain. Every slice was thaw mounted on microscopic

glass slides (SuperFrost Plus Faust, Switzerland). For the detection of cFos expression, frozen sections were air-dried at room temperature for 1 h and rehydrated in PBS. Unspecific binding was blocked by 2 h incubation in 1.5 % normal donkey serum. The primary antibody (1:5000, rabbit anti cFos, Ab-5, Calbiochem) was applied for 48 h at 4 °C. Sections were incubated with the secondary antibody (1:10’000, biotinylated donkey anti rabbit, Jackson 711-065-152) for 2 h at room temperature. After incubation in ABC (Vectastain-Elite ABC Kit, Vector Laboratories), followed by 0.05 % DAB solution (in 0.05 M Tris-HCl with 0.009 % H_2O_2 and color enhancement with 0.04 % $NiCl_2$*$6H_2O$ and 0.08 % $CoCl_2$*$6H_2O$), the sections were dehydrated in graded alcohols, cleared in xylene and fixed with entellan. The rat brain atlas of Swanson was used to localize the cFOS expressing neurons (Swanson, 2004). In 3 adjacent sections cFos positive cells were counted manually ca. at Bregma -13.76 in the AP and the NTS by a treatment blinded investigator.

AP-lesion surgery

AP-lesion was conducted similar to a thermal lesion approach described previously, except that the AP was removed by vacuum aspiration to reduce damage in the NTS (Lutz et al. 1998). Surgery success and specificity was verified functionally and histologically. The functional test was the absence of amylin induced satiation, which depends on an intact AP, and the presence of CCK induced satiation, which depends on intact vagal afferents (Lutz et al. 1998; Ruttimann et al. 2009).16 h food deprived rats were IP injected with amylin (10 µg/kg, Bachem AG, Bubendorf, Switzerland), CCK-8 (4 µg/kg, Bachem AG) or saline, respectively in a cross over design. AP-lesioned animals that did not reduce 30 min post-CCK application food intake by at least 30% were excluded due to a damaged vagus nerve (6 of 24 animals; food intake: sham NaCl 4.2 ± 0.3 g, sham CCK 2.1 ± 0.4 g, AP-lesion NaCl 2.9 ± 0.3

g, AP lesion CCK 0.9 ± 0.3 g). AP-lesioned animals that reduced 2 h post-amylin application food intake by more than 15% were excluded due to incomplete AP-lesion (7 of 24 animals; food intake: sham NaCl 8.6 ± 0.6 g, sham amylin 6.5 ± 0.5 g, AP-lesion NaCl 7.6 ± 0.6 g, AP lesion amylin 8.3 ± 0.7 g). Histological validation of the surgery included a test for the anterograde labeling capacity of vagal afferents to the NTS (Ruttimann et al. 2009). Rats were IP injected with 1 mg fluorogold (Fluorochrome, Denver, USA) in 1 ml saline 3 days before their brains were excised and fixed by a pH-switch protocol described by Khan et al (Khan et al. 2007). Brain sections were cut as described above, slides air dried and fixed with glycerol. An observer blind to surgery quantified the extent of the AP-lesion and counted fluorogold labeled neurons in the NTS. Only animals with histologically confirmed AP-lesion and similar numbers of fluorogold positive NTS neurons as sham lesioned animals were included for the final statistical analysis.

Capsaicin vagotomy

The local vagal capsaicin treatment was performed as previously described (Raybould and Tache, 1988). Surgery success and specificity was verified functionally and histologically. The functional test was the absence of CCK induced satiation, as described above. Capsaicin treatment was considered successful if CCK reduced eating 30 min post application by less than 30% compared to controls (3 of 13 animals excluded; food intake: sham NaCl 4.8 ± 0.2 g, sham CCK 2.5 ± 0.3 g, Capsaicin-treated NaCl 3.9 ± 0.g g, Capsaicin-treated CCK 3.7 ± 0.3 g). Histological validation of the surgery included a test for the anterograde labeling capacity of vagal afferents to the NTS as described above. Only animals with histologically confirmed fluorogold staining in the AP (as injection control) and decreased fluorogold staining in the NTS (due to damaged vagal afferents) were included into the final statistical analysis.

Conditioned taste aversion

Water access is restricted during the 14 days of the conditional taste aversion test. Importantly animals received access to water or saccharine specific to the experimental day as specified below for a 30 min period at dark onset. In the first 4 days of the experiment rats received access to two water bottles to adapt animals to the drinking schedule. On day 5 rats received access to two bottles saccharine (0.3 % in tap water, Sigma). Saccharine intake was equal between experimental groups on day 5 thereby showing equal preference for saccharine intake. Following the 30 min access to saccharine, amino acids (IG, 6.7 mmol/kg; IV 2 mmol/kg) or LiCl (IP, 76 mg/kg, Sigma), which was used as positive control, were administered. This conditioning paradigm was repeated on days 8 and 11; in between, i.e. on days 6-7, 9-10 and 12-13, respectively, water was offered as described for days 1-4. On Day 14 rats were presented one bottle of water and one bottle of 0.3 % saccharine for 30 min at dark onset. Rats were offered water 4 h after dark onset for a 45 min period every day to ensure proper hydration. Liquid intake was measured throughout the entire experiment by weighting the bottles.

Images

Stomach images were captured with a digital camera (D50, Canon), brain slice images with the microscope (Axio Imager 2, Zeiss). Images were not altered except for minor adjustments in brightness and contrast. Fluorogold images were black/white inverted for better visibility.

Statistics

Rats were randomly allocated into treatment and/or surgical group. Application order was randomized. Results are presented as mean ± standard error of the mean. The data were analyzed using Graph Pad Prism 5.0.

Results:

Identification of the most potent oral anorectic amino acids

To identify the most potent anorectic individual amino acid, we administered isomolar doses of all 20 proteogenic amino acids individually to rats via the physiological gastrointestinal route and measured subsequent food intake. The amino acids were administered by gavage at an equal dose of 6.7 mmol/kg, corresponding in the case of Glu to 1g/kg and 27% of average daily Glu intake. Surprisingly the anorectic response induced by individual amino acids did not follow a rank order according to side chain structure, energy content, nutritional necessity (essential) or role as neurotransmitter (-precursor), but rather revealed a unique role for Arg, Lys and Glu (Fig. 1A). Each of these three amino acids significantly reduced food intake in the first hour after administration, whereas all other amino acids had no significant effect compared to water control (Fig. 1A). In the long term only Arg and Lys decreased food intake, whereas Glu treated animals compensated for the initial decrease in food intake in the following 48 h (Fig. 1B). Consistent with previous studies, oral Trp had a small effect on food intake which, however, was statistically not significant when testing for multiple comparisons (Fig. 1A, unpaired two-tailed t-test, $p=0.03$, Ng and Anderson, 1992); the other aromatic amino acids Phe or Tyr did not inhibit food intake as previously shown (Bialik et al. 1989). Meal pattern analysis indicated that oral Arg, Lys and Glu administration reduced eating only by an effect on the first meal size while the latency to meal initiation, meal number, meal duration or time between meals did not differ (Fig. 1C-F). Additional control experiments excluded that pH, osmolarity or the feeding state, hence the presence of nutrients, altered the anorectic effect of Lys (Fig.S1); dose response studies indicated a plateauing of the anorectic effect at 4.7 to 5.4 mmol/kg (Fig. S2A-C). Drinking behavior was not altered by Arg and Lys, but Glu reduced water intake in a dose dependent manner (Fig. S2D-F). These findings are

striking because they identify Arg, Lys and Glu as the individual amino acids with the strongest anorectic effect.

Neuronal pathways mediating the anorectic effect of Arg, Lys and Glu

Eating behavior is ultimately controlled by the brain, which integrates a large array of sensory inputs projecting to specific areas (Berthoud, 2002). Therefore we investigated where in the brain Arg, Lys and Glu triggered neuronal activity by assessing the number of cells expressing cFOS, a neuronal activity marker induced by increases in intracellular Ca^{2+} (Watts et al. 2006). Upon gavage with these three amino acids, an increased number of cFOS positive cells were detected in the nucleus of the solitary tract (NTS) and the area postrema (AP); two areas known to mediate the effect of specific anorectic signals (Fig. 2). Importantly, the AP is not protected by the blood brain barrier such that it may directly sense blood borne signals, and the NTS is the main projection site of vagal afferents (Morton et al. 2006). In contrast, the nucleus accumbens or the hypothalamus (arcuate nucleus, paraventricular nucleus of the hypothalamus, lateral hypothalamic area) did not display increased cFOS expression at this time point. To discriminate whether the anorectic effect of Arg, Lys and Glu was mediated via the AP or vagal afferents, we surgically lesioned both routes. The AP was vacuum aspirated, whereas vagal afferents were chemically lesioned by local sub-diaphragmatic capsaicin application. Surgery specificity and success was validated functionally and histologically (Fig. 3A). Arg and Glu lost their anorectic effect in AP-lesioned animals, but not in capsaicin-treated animals (Fig. 3B-C). Lys, on the other hand, showed an opposite response to the surgical interventions because its anorectic effect was dependent on intact vagal afferents but not on intact AP (Fig. 3). These findings suggest that the

anorectic response to Arg and Glu is induced centrally in the area postrema, whereas Lys response is relayed from the periphery to the NTS by abdominal vagal afferents.

Satiation effects are dissociated from gastrointestinal actions

Vagal afferents fire also in response to gastric distension, which may contribute to meal-ending satiation (Woods, 2009). Apart from the ingestion of food and water, gastric distension can also be due to gastric secretion or delayed gastric emptying, the latter described to depend mainly on the gastric caloric content (Camilleri, 2006). Arg, Lys and Glu increased gastric distension 0.5 h after their application, an effect which coincided with the time of decreased food intake (Fig. 4A). The increase in gastric volume by Arg and Lys administration appeared to be mediated by acidic gastric secretion as the alkaline tide increased plasma albumin-, and decreased plasma Cl^- concentration (Tab. S1). Gastric emptying was delayed by Lys and Glu, as measured by phenol red retention in the stomach (Fig. S3A). For all three amino acids, wet weight of the stomach and/or cecum was still increased 1.5 h after amino acid administration (Fig. 4B). These findings demonstrate that specific amino acids differentially modulate gastrointestinal function, an important finding in light of the caloric focus in this field (Camilleri, 2006). We next tested whether the gastric and anorectic responses to Arg, Lys and Glu are functionally linked by bypassing the gastrointestinal lumen through intravenous amino acid administration. The dose of 2 mmol/kg was selected, because it led to similar changes in plasma amino acid concentration as the oral dose (6.7 mmol/kg) given by gastric gavage (Tab. S2). Intravenous Arg, Lys and Glu reduced food intake similar as after oral application, but only intravenous Glu had an effect on gastric function (Fig. 5, Fig. S3B, Tab. S2). However, in AP-lesioned animals Glu delayed gastric emptying, but did not inhibit food intake (Fig. 3; Fig. S4). These data suggest

that all three amino acids Arg, Lys and Glu, inhibit food intake through circulation and that their effect on gastrointestinal function can be dissociated from their anorectic effect.

Finally we tested if the three amino acids cause visceral discomfort. Therefore we conducted a conditioned taste aversion test after Arg, Lys and Glu administration by the intragastric respectively intravenous route. Oral Glu treatment did not alter the innate preference to saccharine of rats, whereas oral Arg and Lys did induce conditioned taste aversion (Fig. 6A). If Arg and Lys were given intravenously animals did not reduce their saccharine intake (Fig. 6B). These data suggests that the gastrointestinal function changes caused by oral Arg and Lys, but not by intravenous Arg and Lys, induced conditioned taste aversion. Importantly the anorectic effect of Arg and Lys was equally prominent after oral or intravenous application suggesting limited importance of visceral discomfort for their anorectic effect.

Discussion:

Our study establishes a novel role of the amino acids Arg, Lys and Glu in the control of food intake and gastrointestinal function. This expands the existing concept of gastrointestinal function modulation through the caloric content of meals by adding specific amino acids as novel calorie-independent signals (Camilleri, 2006). By inducing gastric secretion these amino acids may favor acid-mediated denaturation of proteins and, by delaying gastric emptying, extend the time for enzymatic protein digestion. Hence, we suggest that the gastrointestinal tract recruits appropriate digestive capacity to cope with a high protein load based on specific amino acid detection. Future work should more systematically test the effect of individual amino acids on gastrointestinal function with an emphasis on translating animal findings to humans. Gastric distension is a mechanical stimulus, transmitted by vagal afferent to the NTS that may contribute to the control of food intake (Schwartz, 2006; Woods, 2009; Davis et al. 1993).Our experiments did however not support such a nutrient unspecific gut-brain axis and showed a clear dissociation of the gastric and the behavioral responses to Arg, Lys and Glu as schematically represented in Fig. 7. In agreement, gastric distensions in balloon inflation studies did not inhibit food intake in rats and humans (Oesch et al. 2006).

Our data highlight the importance of circulating amino acids for the control of food intake and we identified Arg, Lys and Glu as the most effective ones, at least at isomolar doses. Naturally a complete dose-response relationship for all twenty amino acids would be desirable, nevertheless the here used single dose approach enabled the identification of the most potent anorectic amino acids. All three induced satiation by reducing first meal size, but not meal duration, unlike classical satiation-inducing peptides such as CCK or amylin

which proportionally reduce meal size and duration (Woods, 2009). Interestingly, the observed amino acid specificity is in marked contrast to the previously emphasized unique role of the amino acid Leu, which inhibits food intake by regulating mTOR signaling when administered directly to the third brain ventricle (Cota et al. 2006; Morrison et al. 2007). Indeed, consistent with others who showed that physiological changes in plasma Leu concentration do not lead to a reduction of food intake, our data also do not support a role of peripheral Leu for food intake control (Laviano et al. 2006; Zhang et al. 2007; Purpera et al. 2012; Nassl et al. 2011; Morrison et al. 2012). Importantly, so far no systematic study on the effect of the 20 proteogenic amino acids had been published (Fig. 1A). Here, we unravel an amino acid specificity that can be interpreted either as an evolutionary reductive strategy to detect the few amino acids that most accurately indicated protein supply or as a strategy to detect protein quality (Lamb, 2012). Supporting the latter possibility, high protein diets of different sources differentially alter satiation in rodents and humans (Faipoux et al. 2006; Anderson et al. 2004; Pichon et al. 2008).

Interestingly two different afferent pathways respond specifically to one of the candidate amino acids. This redundancy may ensure accurate detection of protein intake *in vivo*. Others have already proposed the existence of a Lys sensor located on vagal afferents innervating the hepatic portal vein. However, the molecular identity of the sensor remained obscure (Torii K & Niijima, 2001). Similarly the molecular mechanism of Arg and Glu sensing by area postrema neurons is unknown, but other previously reported neurons able to sense specific amino acids (Cota et al. 2006; Blouet & Schwartz, 2012; Karnani et al, 2011). We identified here three novel amino acids as potent modulator of short-term food intake and suggest their neuronal site of action. They can contribute to the anorectic effect of a high

protein diet as Arg, Lys and Glu plasma concentrations were shown to raise proportionally to the protein content of a meal (Peters & Harper, 1987). Analogously dietary Arg supplementation was shown to reduce body weight gain in rats (Fu et al, 2005). Based on the aversive response rat exhibit to diets depleted from essential amino acids, we expect a diet depleted from Arg to be rejected but we are currently not aware of such a study (Morrison et al, 2012). One can speculate that a diet supplemented or partially restriction of the three candidate amino acids may mimic the effect of a high respectively of a low protein diet on feeding behavior. Taken together we propose that the central nervous system detects dietary protein content and quality to control food intake by sensing specific circulating amino acids, in particularly Arg and Glu via the AP and Lys via vagal afferents (Fig. 7).

References:

Anderson GH, Tecimer SN, Shah D & Zafar TA (2004). Protein Source, Quantity, and Time of Consumption Determine the Effect of Proteins on Short-Term Food Intake in Young Men. *J Nutr* **134**, 3011–3015.

Berthoud HR (2002). Multiple neural systems controlling food intake and body weight. *Neurosci Biobehav Rev* **26**, 393–428.

Bialik RJ, Li ET, Geoffroy P & Anderson GH (1989). Route of delivery of phenylalanine influences its effect on short-term food intake in adult male rats. *J Nutr* **119**,1519–1527.

Blom W, Lluch A, Stafleu A, Vinoy S, Holst JJ, Schaafsma G & Hendriks HF (2006). Effect of a high-protein breakfast on the postprandial ghrelin response. *Am J Clin Nutr* **83**,211–220.

Blouet C & Schwartz GJ (2012). Brainstem Nutrient Sensing in the Nucleus of the Solitary Tract Inhibits Feeding. *Cell Metab* **7**, 579-587.

Camilleri M (2006). Integrated upper gastrointestinal response to food intake. *Gastroenterology* **131**, 640–658.

Cota D, Proulx K, Smith KA, Kozma SC, Thomas G, Woods SC & Seeley RJ (2006). Hypothalamic mTOR Signaling Regulates Food Intake. *Science* **312**, 927–930.

Davis JD, Smith GP& Miesner J (1993). Postpyloric stimuli are necessary for the normal control of meal size in real feeding and sham feeding rats. *Am J Physiol* **265**, 888-895.

Faipoux R, Tome D, Bensaid A, Morens C, Oriol E, Bonnano LM & Fromentin G (2006). Yeast proteins enhance satiety in rats. *J Nutr* **136**, 2350–2356.

Fromentin G, Darcel N, Chaumontet C, Marsset-Baglieri A, Nadkarni N & Tome D (2012). Peripheral and central mechanisms involved in the control of food intake by dietary amino acids and proteins. *Nutr Res Rev* **25**, 29–39.

Fu WJ, Haynes TE, Kohli R, Hu J, Shi W, Spencer TE, Carrol RJ, Meininger CJ & Wu G (2005). Dietary L-arginine supplementation reduces fat mass in Zucker diabetic fatty rats. *J Nutr* **135**, 714-721

Karnani MM, Apergis-Schoute J, Adamantidis A, Jensen LT, de Lecea L, Fugger L& Burdakov D (2011). Activation of central orexin/hypocretin neurons by dietary amino acids. *Neuron* **72**, 616-629.

Khan AM, Ponzio TA, Sanchez-Watts G, Stanley BG, Hatton GI & Watts AG (2007). Catecholaminergic control of mitogen-activated protein kinase signaling in paraventricular neuroendocrine neurons in vivo and in vitro: a proposed role during glycemic challenges. *J Neurosci* **27**, 7344–7360.

Lamb RF (2012). Amino acid sensing mechanisms: an Achilles heel in cancer? *FEBS J* **279**, 2624–2631.

Laviano A, Meguid MM, Inui A & Rossi-Fanelli F (2006). Role of leucine in regulating food intake. *Science* **313**, author reply 1236-1238.

L'Heureux-Bouron D, Tome D, Bensaid A, Morens C, Lacroix M, Huneau JF& Fromentin G (2004). Preabsorptive factors are not the main determinants of intake depression induced by a high-protein diet in the rat. *Physiol Behav* **81**, 499–504.

L'Heureux-Bouron D, Tome D, Rampin O, Even PC, Larue-Achagiotis C & Fromentin G (2003). Total subdiaphragmatic vagotomy does not suppress high protein diet-induced food intake depression in rats. *J Nutr* **133**, 2639–2642.

Lutz TA, Senn M, Althaus J, Del Prete E, Ehrensperger F & Scharrer E (1998). Lesion of the area postrema/nucleus of the solitary tract (AP/NTS) attenuates the anorectic effects of amylin and calcitonin gene-related peptide (CGRP) in rats. *Peptides* **19**, 309–317.

Morrison CD, Reed SD & Henagan TM (2012). Homeostatic regulation of protein intake: in search of a mechanism. *Am J Physiol Regul Integr Comp Physiol* **302**, 917-928.

Morrison CD, Xi X, White CL, Ye J, Martin RJ (2007). Amino acids inhibit Agrp gene expression via an mTOR-dependent mechanism. *Am J Physiol Endocrinol Metab* **293**, 165-171.

Morton GJ, Cummings DE, Baskin DG, Barsh GS & Schwartz MW (2006). Central nervous system control of food intake and body weight. *Nature* **443**, 289–295.

Nassl AM, Rubio-Aliaga I, Sailer M & Daniel H (2011). The intestinal peptide transporter PEPT1 is involved in food intake regulation in mice fed a high-protein diet. *PLoS One* **6**, e26407.

Ng LT & Anderson GH (1992). Route of administration of tryptophan and tyrosine affects short-term food intake and plasma and brain amino acid concentrations in rats. *J Nutr* **122**, 283–293.

Niv Y, Abu-Avid S & Neumann G (1993). Further applications of blood gas analysis to gastric acidity determination. *Clin Chim Acta* **215**, 9–19.

Oesch S, Ruegg C, Fischer B, Degen L & Beglinger C (2006). Effect of gastric distension prior to eating on food intake and feelings of satiety in humans. *Physiol Behav* **87**, 903–910.

Peters JC & Harper AE (1987). Acute effects of dietary protein on food intake, tissue amino acids, and brain serotonin. *Am J Physiol* **252**, 902-914

Pichon L, Potier M, Tome D, Mikogami T, Laplaize B, Martin-Rouas C & Fromentin G (2008). High-protein diets containing different milk protein fractions differently influence energy intake and adiposity in the rat. *Br J Nutr* **99**, 739–748.

Potier M, Darcel N & Tome D (2009). Protein, amino acids and the control of food intake. *Curr Opin Clin Nutr Metab Care* **12**, 54–58.

Purpera MN, Shen L, Taghavi M, Munzberg H, Martin RJ, Hutson SM & Morrison CD (2012). Impaired branched chain amino acid metabolism alters feeding behavior and increases orexigenic neuropeptide expression in the hypothalamus. *J Endocrinol* **212**, 85–94.

Raybould HE & Tache Y (1988). Cholecystokinin inhibits gastric motility and emptying via a capsaicin-sensitive vagal pathway in rats. *Am J Physiol* **255**, 242-246.

Ruttimann EB, Arnold M, Hillebrand JJ, Geary N & Langhans W (2009). Intrameal hepatic portal and intraperitoneal infusions of glucagon-like peptide-1 reduce spontaneous meal size in the rat via different mechanisms. *Endocrinology* **150**, 1174–1181.

S J Rune (1966). Comparison of the rates of gastric acid secretion in man after ingestion of food and after maximal stimulation with histamine. *Gut* **7**, 344–350.

Schwartz GJ (2006). Integrative capacity of the caudal brainstem in the control of food intake. *Philos Trans R Soc Lond B Biol Sci* **361**, 1275–1280

Swanson LW (2004). *Brain maps III. Structure of the rat brain / an atlas with printed and electronic templates for data, models and schematics*. 3rd edn. Elsevier Academic Press, Amsterdam.

Tome D, Schwarz J, Darcel N & Fromentin G (2009). Protein, amino acids, vagus nerve signaling, and the brain. *Am J Clin Nutr* **90**, 838-843.

Torii K & Niijima A (2001). Effect of lysine on afferent activity of the hepatic branch of the vagus nerve in normal and L-lysine-deficient rats. *Physiol Behav* **72**, 685-690.

Tsurugizawa T, Uematsu A, Nakamura E, Hasumura M, Hirota M, Kondoh T, Uneyama H & Torii K (2009). Mechanisms of neural response to gastrointestinal nutritive stimuli: the gut-brain axis. *Gastroenterology* **137**, 262–273.

Watts AG, Khan AM, Sanchez-Watts G, Salter D & Neuner CM (2006). Activation in neural networks controlling ingestive behaviors: what does it mean, and how do we map and measure it? *Physiol Behav* **89**, 501–510.

Woods SC (2009). The control of food intake: behavioral versus molecular perspectives. *Cell Metab* **9**, 489–498.

Zhang Y, Guo K, LeBlanc RE, Loh D, Schwartz GJ & Yu YH (2007). Increasing dietary leucine intake reduces diet-induced obesity and improves glucose and cholesterol metabolism in mice via multimechanisms. *Diabetes* **56**, 1647–1654.

Author Contribution: All authors contributed to study design, data interpretation and manuscript revision. B.H. contributed to data collection. J.J. performed all experiments and surgeries and wrote the paper with the help of T.A.L. and F.V. All aspects of the project were supervised by T.A.L. and F.V. All authors approved the final version of the manuscript and all authors qualifying for authorship are listed.

Acknowledgments and Disclosure: All authors declare no competing financial, personal, or professional interest that could be construed to have influenced this paper. We want to thank Daniel Tomé for discussions and Daria Stöcker, Annika Donauer and Myrtha Arnold for technical advice. This work was supported by Zurich Center of Integrative Human Physiology (ZHIP), the Swiss NSF grant 31-130471/1 to FV and 31003A-138 246 to TAL.

Figures & Figure Legends:

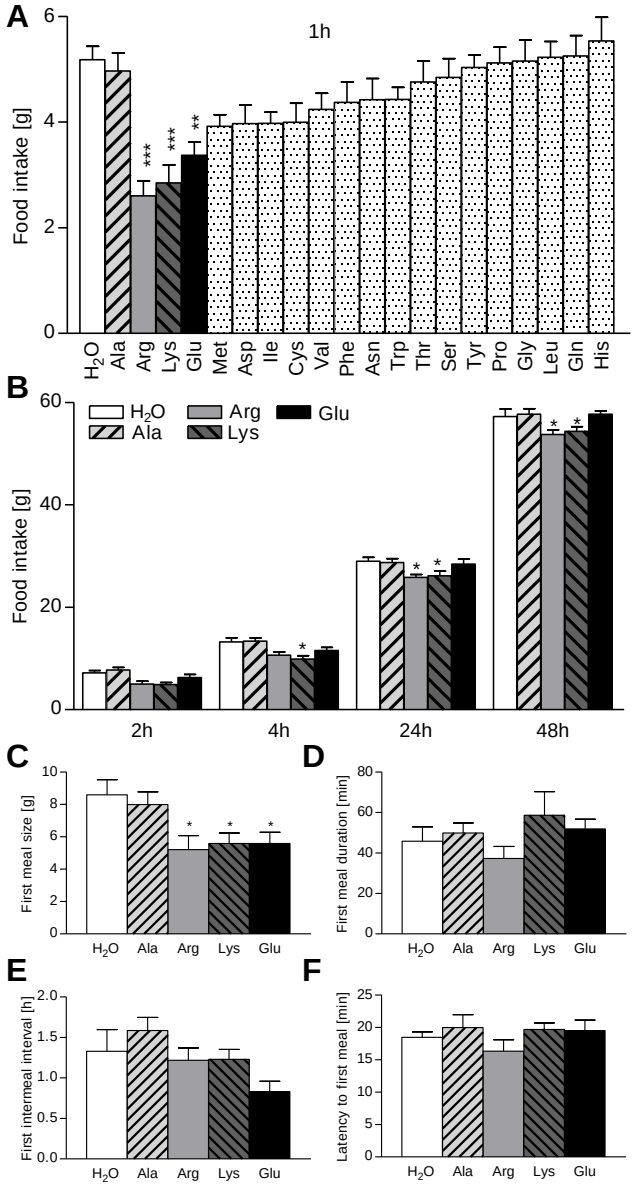


Figure 1. Intragastric Arg, Lys and Glu inhibited food intake most potently of all proteogenic amino acids. After 12 h food deprivation rats were gavaged with isomolar doses of individual amino acids (6.7 mmol/kg, which in the case of Glu corresponds to 1 g/kg and to 27 % of the daily average Glu intake) and their subsequent food intake measured for 1 (A), 2, 4, 24 and 48 h (B); n = 12, mean ± sem; (A) unpaired one-way ANOVA, Dunnett post test; (B) unpaired two-way ANOVA, Bonferroni post test; *p < 0.05, **p < 0.01, ***p < 0.001. (C-F) After 16 h food deprivation rats were gavaged with individual amino acids (6.7

mmol/kg) and their subsequent meal pattern analyzed in an automated BioDaq system; n = 10, mean ± sem; unpaired one-way ANOVA, Dunnett post test; *p < 0.05.

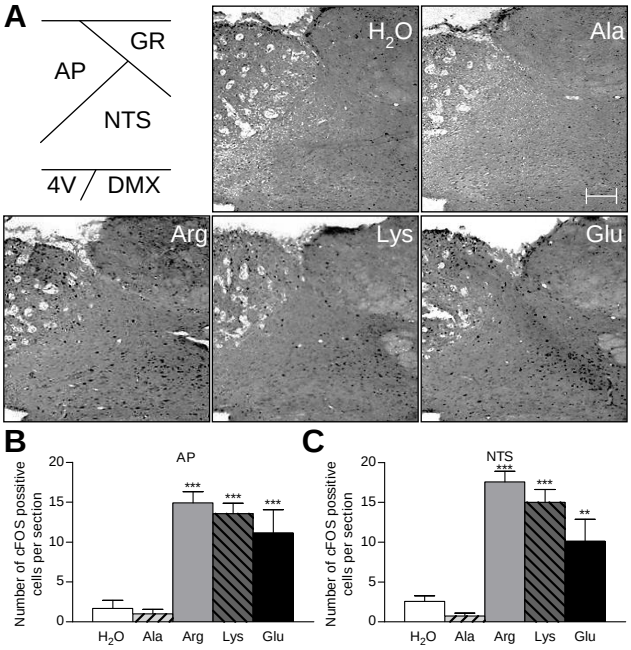


Figure 2; Intragastric Arg, Lys and Glu induced neuronal activity in the brainstem. After 16 h food deprivation, rats were gavaged with isomolar doses (6.7 mmol/kg) of individual amino acids and transcardially perfused with PFA 2 h later. Animals had no access to food between gavage and transcardial perfusion. Different brain areas were analyzed for cFOS expression. (A) Representative images showing cFOS positive cells in the AP and the NTS (located -13.76 from bregma). Scale bar, 100 µm. AP indicates the area postrema, NTS – nucleus of the solitary tract, 4V – 4th ventricle, DMX – dorsal motor nucleus vagus nerve, GR – gracile nucleus. Quantification of cFOS positive cells in the AP (B) and NTS (C); n = 6-8, mean ± sem; unpaired one-way ANOVA, Dunnett post test; **p < 0.01, ***p < 0.001.

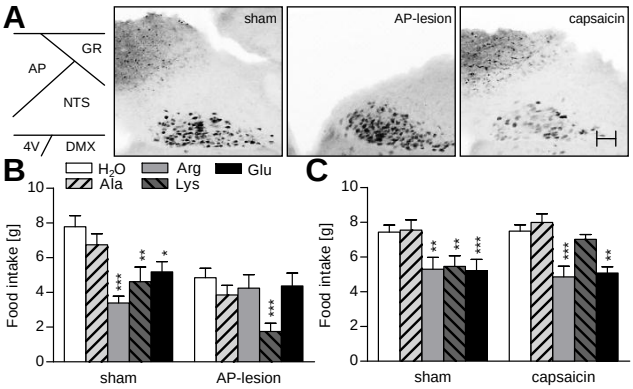


Figure 3. The AP mediated the anorectic effect of Arg and Glu, whereas abdominal vagal afferents are necessary for the anorectic effect of Lys. (A) Histological surgery validation showed complete removal of the AP and an intact vagus nerve, visualized by retrograded tracing of IP injected fluorogold to the NTS (primary vagal projection site), in AP-lesioned animals. Capsaicin treatment lesioned the vagus nerve and consequently less fluorogold reached the NTS in respective animals. The AP showed fluorogold labeling due to the absence of the blood brain barrier. Location -13.76 from bregma, 4V indicates the 4th ventricle, DMX – dorsal motor nucleus vagus nerve, GR – gracile nucleus, scale bar 100 μm. After 16 h food deprivation AP-lesioned (B), capsaicin-treated (C) and the respective sham animals were gavaged with individual amino acids (6.7 mmol/kg) and their subsequent food intake measured for 1 h; n = 7-11, mean ± sem; repeated measures two-way ANOVA, Bonferroni post test; *p < 0.05, **p < 0.01, ***p < 0.001.

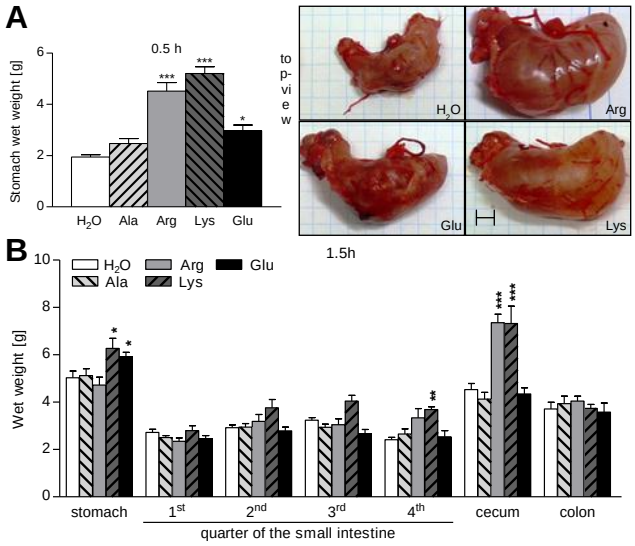


Figure 4. Arg, Lys and Glu induced gastric distension by distinct mechanisms. (A) After 16 h food deprivation rats were gavaged with an isovolumic (2 ml) dose of individual amino acids (6.7 mmol/kg), 30 min later their stomach was excised and weighted; n = 6, mean ± sem; unpaired one-way ANOVA, Dunnett post test; *p < 0.05, ***p < 0.001. Representative images are shown; scale bar, 4 mm. (B) After 16 h food deprivation rats were gavaged with individual amino acids (6.7 mmol/kg), 30 min later received access to 3 g rat chow and 1.5 h post-administration the gastrointestinal tract was excised and the wet weight measured; n = 6, mean ± sem; unpaired two-way ANOVA, Bonferroni post test; *p < 0.05, **p < 0.01, ***p < 0.001.

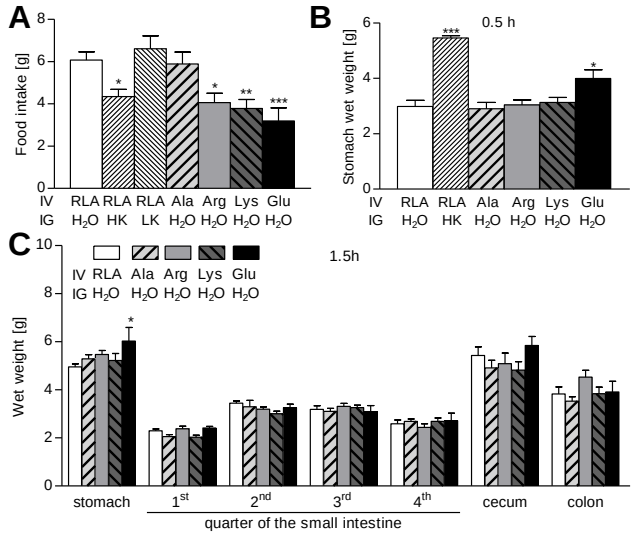


Figure 5. Intravenous Arg, Lys and Glu inhibited food intake, but only intravenous Glu delayed gastric emptying. (A) After 16 h food deprivation rats were anesthetized, received a Ringer lactate (RLA) or amino acid (2 mmol/kg) injection into the lateral tail vein, were gavaged with water or amino acid (HK, 6.7 mmol/kg Lys; LK, 2 mmol/kg Lys) and their subsequent food intake was measured for 1 h; n = 9-11, mean ± sem; unpaired one-way ANOVA, Dunnett post test; *p < 0.05, **p < 0.01, ***p < 0.001. (B) After 16 h food deprivation rats were anesthetized, received a Ringer lactate (RLA) or amino acid (2 mmol/kg) injection into the lateral tail vein, were gavaged with water or amino acid (HK, 6.7 mmol/kg Lys; LK, 2 mmol/kg Lys), 30 min later their stomach was excised and weighted; n = 6, mean ± sem; unpaired one-way ANOVA, Dunnett post test; *p < 0.05, ***p < 0.001. (C) After 16 h food deprivation rats were anesthetized, received a Ringer lactate (RLA) or amino acid (2 mmol/kg) injection into the lateral tail vein, were gavaged with water, 30 min later received access to 3 g rat chow and 1.5 h post-administration the gastrointestinal tract was excised and the wet weight measured; n = 6, mean ± sem; unpaired two-way ANOVA, Bonferroni post test; *p < 0.05.

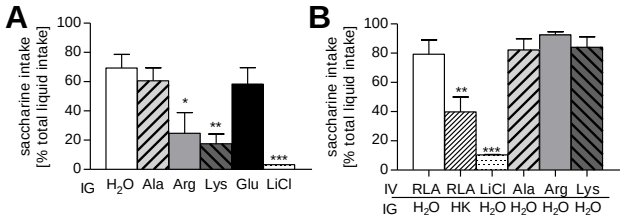


Figure 6. Conditioned taste aversion test following different routes of amino acid administration. (A) Rats received an amino acid (6.7 mmol/kg) or water gavage during the conditioning days. (B) Rats received an amino acid (2 mmol/kg) or Ringer lactate (RLA) injection into the lateral tail vein and were gavaged with water or amino acid (HK, 6.7 mmol/kg Lys) during the conditioning days. IP injected LiCl is the positive control. n = 6 (LiCl n = 3), mean ± sem; unpaired one-way ANOVA, Dunnett post test; *p < 0.05, **p < 0.01, ***p < 0.001.

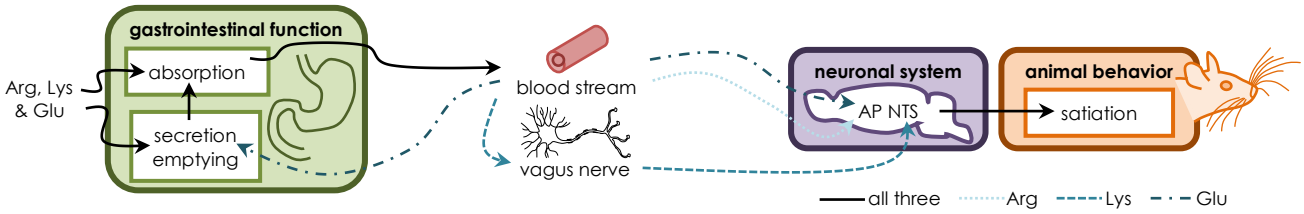


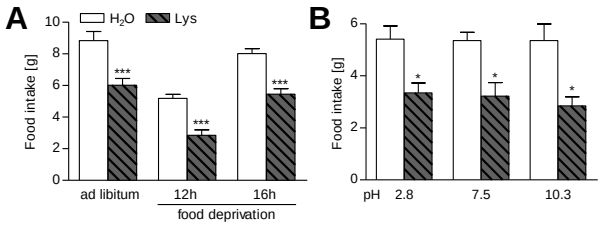
Figure 7. Schematic representation of main findings. Arg, Lys and Glu were identified as the most potent oral anorectic amino acids. After gastric delivery, they specifically stimulate gastric secretion and/or delay gastric emptying thereby facilitating digestion. After absorption only Glu can inhibit gastric emptying. Importantly all three amino acid also inhibit food intake when administered intravenously but by different neuronal mechanism. Lys is detect by vagal afferents projecting to the NTS, whereas Arg and Glu centrally in the area postrema - a brain areas not protected by the blood barrier. Thus, Arg, Lys and Glu selectively impact on food processing and intake suggesting them as direct sensory input to assess dietary protein content and quality.

Supplementary Material

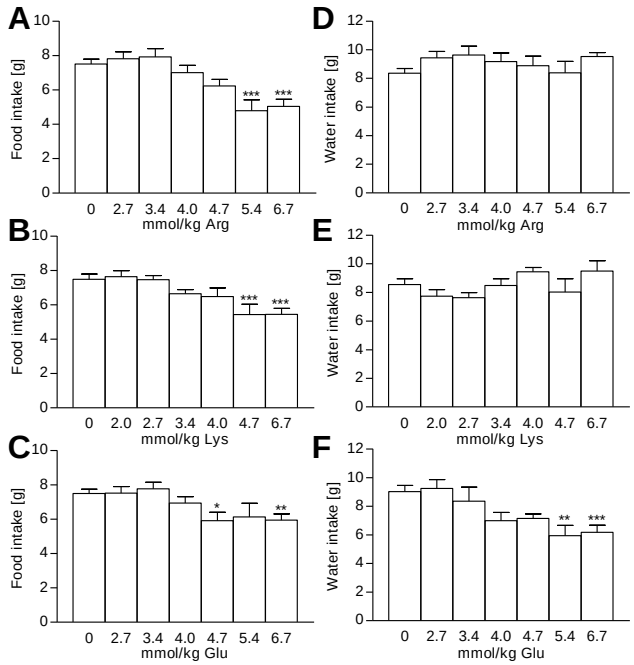
Specific amino acids inhibit food intake via the area postrema or vagal afferents.

Josua Jordi[1,2,3], Brigitte Herzog[1,3], Simone M. R. Camargo[1,3], Christina N. Boyle[2,3], Thomas A. Lutz[2,3,*], François Verrey[1,3,*]

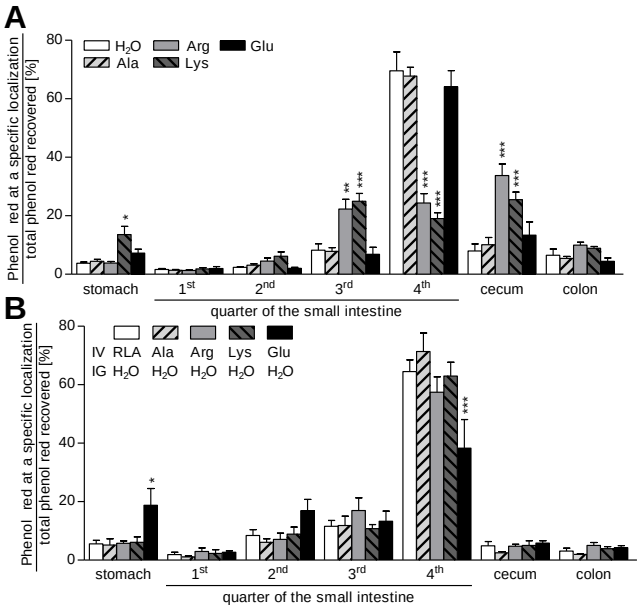
Supplementary Figures



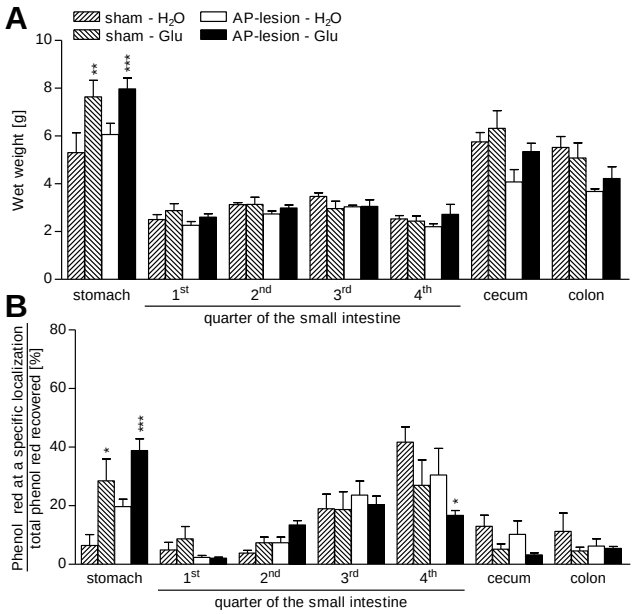
Supplementary Figure 1. The anorectic effect of Lys was independent to feeding state and pH. (A) Rats were fed ad libitum, food deprived for 12 h or 16 h and gavaged with isomolar doses (6.7 mmol/kg) of Lys at dark onset. Food deprivation was used to synchronize food intake and thereby reduce variation. Food intake of the 12 h or 16 h food deprived animals was measured for 1 h (n = 10), and the ad libitum fed group for 6 h (n = 20); mean ± sem; unpaired two-tailed student t-test; ***$p < 0.001$. (B) After 12 h food deprivation rats were gavaged with 6.7 mmol/kg Lys adjusted to pH 2.8, 7.5 and 10.3 (n = 7) and their subsequent food intake measured for 1 h; mean ± sem; unpaired two-tailed student t-test; *$p < 0.05$.



Supplementary Figure 2. Dose response of Arg, Lys and Glu. After 16 h food deprivation rats were gavaged with different doses (as indicated) of individual amino acids and their subsequent (A-C) food and (D-F) water intake measured for 1 h; n = 10, mean ± sem; unpaired one-way ANOVA, Dunnett post test; *p < 0.05, **p < 0.01, ***p < 0.001.



Supplementary Figure 3. Arg, Lys and Glu differentially altered gastrointestinal function dependent on route of application. (A) After 16 h food deprivation rats were gavaged with isomolar doses (6.7 mmol/kg) of individual amino acids including phenol red (1.5 mg). Thirty minutes later they received access to 3 g rat chow and 1.5 h post-administration, the gastrointestinal tract was excised and phenol red content quantified; n = 6, mean ± sem; unpaired two-way ANOVA, Bonferroni post test; *p < 0.05, **p < 0.01, ***p < 0.001. (B) After 16 h food deprivation rats were anesthetized, received a Ringer lactate (RLA) or amino acid (2 mmol/kg) injection into the lateral tail vein and were gavaged with water including phenol red (1.5 mg). Thirty minutes later they received access to 3 g rat chow and 1.5 h post-administration, the gastrointestinal tract was excised and the phenol red content quantified; n = 6, mean ± sem; unpaired two-way ANOVA, Bonferroni post test; *p < 0.05, ***p < 0.001.



Supplementary Figure 4. Glu delayed gastric emptying in AP-lesioned animals. (A) After 16 h food deprivation rats were gavaged with water or 6.7 mmol/kg Glu, 30 min later received access to 3 g rat chow and 1.5 h post-administration the gastrointestinal tract was excised and the wet weight measured; n = 4-6, mean ± sem; unpaired two-way ANOVA, Bonferroni post test; **p < 0.01, ***p < 0.001. (B) After 16 h food deprivation rats were gavaged with water or 6.7 mmol/kg Glu including 1.5 mg phenol red. Thirty minutes later they received access to 3 g rat chow and 1.5 h post-administration the gastrointestinal tract was excised and phenol red content quantified; n = 4-6, mean± sem; unpaired two-way ANOVA, Bonferroni post test; *p < 0.05, ***p < 0.001.

Supplementary Tables

Supplementary Table 1. Blood plasma parameters 30 min after gavage.

IG[£]	H_2O	Ala	Arg	Lys	Glu
Chloride [mmol/l]	91.0±1.2	92.7±0.9	83.8±1.4[***]	88.3±1.3	94.8±0.9
Albumine [g/dl]	1.56±0.03	1.68±0.02	1.70±0.04[*]	1.75±0.03[**]	1.58±0.04
Sodium [mmol/l]	145.7±0.5	144.8±0.9	146.2±1.3	145.7±0.8	145.4±1.2
Glucose [mg/dl]	209±29	200±17	214±11	211±18	199±13

[£]After 16 h food deprivation rats were gavaged with isomolar doses (6.7 mmol/kg) of amino acids and 30 min later blood collected by heart puncture; n = 6, mean ± sem; unpaired one-way ANOVA, Dunnett post test, *p < 0.05, **p < 0.01, ***p < 0.001.

Supplementary Table 2. Blood plasma parameters 30 min after intravenous administration.

IV[£]	RLA	RLA	Ala	Arg	Lys	Glu
IG	H_2O	HK	H_2O	H_2O	H_2O	H_2O
Lys [µmol/l]	150±13	770±100[***]	178±20	144±8	856±50[***]	127±89
Chloride [mmol/l]	89.8±0.5	90.8±0.5	93.2±0.7	92.8±1.8	93.0±0.7	91.0±1.1
Albumine [g/dl]	2.06±0.04	2.28±0.05[*]	2.07±0.03	1.92±0.09	2.05±0.03	2.10±0.10
Sodium [mmol/l]	148.0±1.2	149.2±2.1	152.0±1.6	145.8±2.2	146.5±1.3	149.8±0.5
Glucose [mg/dl]	135±4	146±3	136±3	134±4	132±3	145±5

[£]After 16 h food deprivation rats were anesthetized, received a Ringer lactate (RLA) or amino acid (2 mmol/kg) injection into the lateral tail vein, were gavaged with water or amino acid (HK – 6.7 mmol/kg Lys) and 30 min later blood was collected from the tongue in short-term anesthetized animals (isoflurane); n = 6, mean ± sem; unpaired one-way ANOVA, Dunnett post test; *p < 0.05, ***p < 0.001.

5. Original Research Article: “Simultaneous assessment of gastric emptying and secretion in rats by a novel computed tomography based method”

This section contains an original research article that was submitted to publication in the American Journal of Physiology in the Section of Gastrointestinal and Liver Physiology in July 2013.

My contribution to this paper includes the study design and data interpretation under the supervision of F. Verrey and T. Lutz. I performed all experiments and wrote the paper with the help of F. Verrey and T. Lutz.

Title: Simultaneous assessment of gastric emptying and secretion in rats by a novel computed tomography based method

Authors: Josua Jordi[1,2,3], François Verrey[1,3,*], Thomas A. Lutz[2,3,*]

Affiliations: [1]Institute of Physiology, University of Zurich, Switzerland; [2]Institute of Veterinary Physiology, University of Zurich, Switzerland; [3]Zurich Center of Integrative Human Physiology, University of Zurich, Switzerland; [*]These authors contributed equally to this work.

Running Head: Quantification of gastric emptying and secretion *in vivo*

Corresponding Author: Thomas A. Lutz, Prof. Dr. med. vet.
Institute of Veterinary Physiology, University of Zürich
Winterthurerstr. 260, CH-8057 Zürich, Switzerland
Phone +41 44 635 88 08 Fax +41 635 89 32
tomlutz@vetphys.uzh.ch

Keywords: gastric emptying, gastric secretion, computed tomography, CCK, Histamine, Glucose

Abstract

Gastric emptying and gastric secretion are two major physiological functions of the stomach. The assessment of these functions in particular in small animals is challenging; no method currently available allows the simultaneous measurement of both functions, and methods used are lethal or invasive and often limited by spatial, temporal or quantitative resolution. Here, we report the establishment and validation of a quantitative non-invasive high-throughput computed tomography based method to measure simultaneously gastric emptying and secretion in rats *in vivo*. The imaging strategy enables to visualize stomach anatomy and quantify stomach volume and stomach contrast agent content. The method was validated by comparing the results to classical lethal methods (stomach phenol red content and stomach wet weight). Additionally, we showed that the use of a mild anesthetic does not interfere with normal gastric emptying and secretion thereby enabling high-resolution temporal studies in the same animal. These combined advantages were applied to reevaluate the impact of cholecystokinin (CCK), histamine and oral glucose solutions on gastric function with high temporal resolution. CCK inhibited gastric emptying completely for 20 min leading to the accumulation of gastric juice in the stomach. The CCK antagonist Devazepide blocked this effect. Histamine stimulated both gastric secretion and delayed emptying. Oral glucose solution emptied at a fix rate of 24-31 cal/min and stimulated gastric secretion. These results confirmed previous observations and added volumetric changes as a new dimension. As computed tomography scanners become broadly available this method is an excellent approach to measure the combined gastric functional readout and to reduce animal interventions.

Introduction

Gastrointestinal (GI) transit, nutrient digestion and absorption are highly controlled functions. Each of them is to some extent autonomously regulated by neuronal and endocrinal mechanisms but also controlled by the central nervous system (3). Based on the multifactorial control mechanism, it is not surprising that impaired GI function is associated with a broad spectrum of pathologies (Diabetes Type 1/2, Parkinson, etc) (10, 17, 21). Due to the multiple involved signals, GI function needs to be studied *in vivo*, ideally with non-invasive methods.

The present study focuses on two main functions of the stomach, i.e. gastric secretion and gastric emptying. Gastric secretions participate in nutrient digestion but acid secretion is also an important component of the body's defense mechanisms. For instance, patients with decreased gastric secretion are more prone to enteric infections by *H. pylori*, *E. coli* or *V. cholera* (15, 21). The rate of gastric emptying determines nutrient release into the small intestine, which is particularly important to maintain postprandial nutrient homeostasis. Our understanding of stomach functions evolved rather slowly due to the lack of suitable *in vivo* methods applicable to high-throughput studies in rodents.

One challenge in measuring stomach secretion and motility is the high speed of these functions thereby requiring methods with high temporal resolution [see recent reviews (7, 22)]. A classical method to assess gastric emptying compares the residual gastric dye content (e.g. radioactive compounds or phenol red) between treatment groups at specific time points (12, 24). This method requires stomach excision and therefore is lethal for the experimental animal. Alternatively, gastric fistulas can be implanted to aspirate gastric content (13, 18). Experimental success depends strongly on fistula positioning, because it determines localization and extent of aspiration. Apart of its invasive nature, surgery may also interfere with normal gastric function. Non-invasive alternatives are paracetamol absorption or breath-tests (^{13}C acetate) (4, 16). Both methods are indirect and are limited by the fact that

unchanged metabolism and absorption kinetics across experimental groups have to be assumed. Currently the most promising avenue might be the use of *in vivo* imaging strategies including ultrasound, positron-emission-tomography or bioluminescence among others (1, 2, 11, 19). However, so far their use remained limited due to difficulties with time- or spatial-resolution, throughput or quantitative measures. Apart from these technical limitations, these imaging strategy are also unable to assess gastric secretion and emptying within the same measurement. Nevertheless they are critical components in the efforts to reduce lethal and invasive animal experiments and thereby important in context of the current 3R (replacement, refine, reduce) goals anchored in most national guidelines on animal welfare.

Advancements in small animal X-ray technology led to a reduction of the scanning times for computed tomography (CT) to few seconds while the image quality increased markedly in spatial resolution (8). Hence, X-ray exposure was markedly reduced enabling several hundred scans per animal before reaching critical doses (>4 Gy for rats). A disadvantage of X-ray-based techniques is the poor absorption of radiation in soft tissue such as the stomach epithelium and intestinal content. This can be compensated by the oral application of contrast agents such as barium sulfate or sodium diatrizoate (SDH). Hence, the quantitative detection of stomach contrast agent content and volume might provide an alternative imaging method to visualize stomach function *in vivo*.

Here, we report the establishment and validation of a quantitative non-invasive high-throughput computed tomography based method to measure simultaneously gastric emptying and secretion in rats *in vivo*. The contrast agent based strategy enables visualization and quantification of the total stomach volume and the residual contrast agent with high temporal resolution. The method was extensively validated using known modulators of gastric emptying and secretion.

Glossary

GI – gastrointestinal

CT – computed tomography

SDH – sodium diatrizoat hydrate (contrast agent)

IP - intraperitonal

SC – subcutaneous

CCK - cholecystokinin

Materials and Methods

Animals

Male Wistar rats (Janvier, France) were group housed (room temperature 21 ± 1° C, artificial 12/12 h light dark cycle, water ad libitum, rat chow ad libitum [3433 Kliba Nafag, Kaiseraugst, Switzerland]). Body weight increased throughout the study by 3-4 g/day. All procedures for rat handling and experimental interventions were according to the Swiss Animal Welfare legislation and approved by the Kantonales Veterinäramt Zürich.

Contrast Agent

Sodium diatrizoate hydrate (SDH, Sigma-Aldrich, Buchs, Switzerland) was solubilized in tap water and adjusted to pH 7.2, if not stated differently. A liquid contrast agent was selected because we assumed more accurate detection of the total stomach volume compared to a solid contrast agent (barium sulfate). We selected SDH instead of Meglumine diatrizoat, because SDH generated a stronger contrast and is less expensive. Both agents are used in clinics (gastrografin) and side effects are at most mild and transitory (FDA rev.10/11, NDC 0270–0445–40).

In vitro quantification of SDH concentration and liquid volume by micro-CT

Different solutions were prepared *in vitro* and of each solution one CT image acquired. First, SDH was diluted to different concentrations (50, 100, 200, 300, 400, 500 mg/ml) with tap water. 120 µl of each of these solutions were used for imaging in a 1.5 ml tube. Second, 125 mg of SDH was dissolved in 0.5, 0.6, 0.7, 0.9, 1.3, 2.1 ml tap water in a 15 ml tube (concentrations of 250, 208, 179, 139, 96, 60 mg/ml). The entire tube and volume was imaged using the CT scanner. Third, 200 mg SDH were dissolved in 1 ml tap water, pH adjusted to 3, 5, 7, 9, 11 with HCl or NaOH respectively and one CT image acquired of each solution in a 1.5 ml tube.

In vivo quantification of SDH concentration and liquid volume by micro-CT

Food deprivation was used to ensure similar initial stomach contents between animals. 4 h food deprived rats were anesthetized with isoflurane (5 %), gavaged with different solutions and immediately scanned by CT under isoflurane anesthesia. In the first set of experiments 2 ml of solutions were orally applied with different SDH concentrations (2 ml/animal; 30, 50, 70, 100, 150, 200 mg/ml; 60, 100 140, 200, 300, 400 mg/animal). In the second set of experiments 200 mg SDH was applied in different volumes of tap water (1, 1.1, 1.2, 1.4, 1.8, 2.5, 3 ml/animal; 200, 181, 167, 143, 111, 80, 67 mg/ml; 200 mg/animal).

mCT validation against the classical phenol red method

4 h food deprived rats received an intraperitonal (IP) injection of CCK-8 (4 μg/kg, Bachem AG, Switzerland) or saline (vehicle), immediately followed by 2 ml gavage of 200 mg/ml SDH and 0.75 mg/ml phenol red (Sigma). Animals were returned to their home cages, but did not have access to water or food. 20 min post-application animals were euthanized using pentobarbital (IP, 100 mg/kg, Kantonsapotheke Zürich, Switzerland) in combination with isoflurane (5 %) for quicker induction. Immediately after anesthesia induction CT images were acquired and the stomach excised. The stomach wet weight was measured and the residual stomach phenol red content extracted and quantified spectroscopically at 560 nm as described before (24).

Stomach function under anesthesia

One groups of 4 h food deprived rats were anesthetized using Zoletil (Zoletil = Tiletamine base : Zolazepam base , IP or subcutaneous (SC), 20 mg/kg, Virbac, Glattbrugg, Switzerland). After anesthesia induction, the rats' eyes were covered with Vitamin A ointment (Kantonsapotheke Zürich), rats gavaged with 2 ml SDH (200 mg/ml) and subsequently imaged immediately after gavage and then every 5 min. Animals were kept on a

heat-plate during the experiment to limit heat loss. The second group was not anesthetized during the experimental time enabling normal gastric function thereby reflecting the non-anesthetized condition. After a specific time window these animals were anesthetized to image one single time point. Hence, each time point is one animal not exposed to anesthesia before and therefor termed endpoint anesthesia. Animals undergoing the endpoint anesthesia protocol were 4 h food deprived, received a gavaged with 2 ml SDH (200 mg/ml) and returned to their home cage, where they did not have access to food or water. After a defined time interval, the animals were anesthetized using 5 % isoflurane and immediately imaged.

Pharmacological modulation of gastric emptying and secretion by CCK and histamine

In the first experiment, 4 h food deprived rats received an IP injection of devazepide (1 mg/kg, Sigma) or vehicle (10 % DMSO 10 % Tween 80 in 0.9 % NaCl, all Sigma). After 10 min rats received an IP injection of CCK-8 (4 μg/kg) or saline and Zoletil (IP, 20 mg/kg). In the second experiment, 4 h food deprived rats received and IP injection of histamine (5 mg/kg, Sigma) or vehicle (10 % DMSO 10 % Tween 80 in 0.9 % NaCl, all Sigma) and Zoletil (IP, 20 mg/kg). In both experiments, eyes were covered with Vitamin A ointment after anesthesia induction, then rats were gavaged with 2 ml SDH (200 mg/ml) and subsequently imaged immediately and every following 5 min. Animals were kept on a heat-plate during the experiment to limit heat loss.

Oral glucose empties at a rate of 24-31 cal/min

4 h food deprived rats received an IP Zoletil injection (20 mg/kg). After anesthesia induction eyes were covered with Vitamin A ointment, rats gavaged with 2 ml of different glucose solutions (0, 0.2, 0.4 g/ml D-(+)-glucose and 200 mg/ml SDH, pH 7.2, all Sigma) and subsequently imaged immediately and every following 5 min. Animals were kept on a heat-plate during the experiment to limit cooling.

Imaging acquisition

Images were acquired using Quantum FX microCT (Perkin Elmer, Massachusetts, USA). Rats were imaged in a prone position using a field of view sized 73 mm. Respiratory gating was used to reduce motion artifacts. The radiation dose was 26 mGy / scan enabling a voxel size of 148 μm^3. Scan time was 34 s / scan.

Image analysis

Images were analyzed using Caliper Analyze 11.0 (Analyze Direct, Kansas, USA). All images were treated equally i.e. no changes to contrast or intensity were applied nor any filter to enhance image quality. Original images were subject to a semi-automatic object extraction algorithm which is part of the software package. Briefly, a seed point was selected within the object of interest which, in our case, was the contrast agent within the stomach; a threshold was set to the intensity 2000 based on the *in vitro* experiments (see Fig. 1A). The algorithm automatically assigned every voxel connected to the seed point above the threshold to the object. The extracted object was visually inspected by a treatment blinded investigator. If the algorithm had falsely assigned voxels within the esophagus or the small intestine to the object, they were manually removed. Subsequently object volume and mean intensity were extracted. The displayed stomach images are 3D-volume renderings of representative stomachs for the specified condition. For the mathematical analysis of the results, see equation section.

Statistics

Rats were randomly allocated to treatment groups, and the order of application was randomized. Results are presented as mean ± standard error of the mean. The data were analyzed using Graph Pad Prism 5.0. Statistical significance between the means was tested using unpaired Student *t*-test or two-way ANOVA and a Bonferroni post-test as appropriate. Differences were considered significant at $P < 0.05$.

Results

In vitro quantification of SDH concentration and liquid volume by micro-CT

To test if SDH can be detected quantitatively we diluted SDH to different concentrations and quantified the signal intensity and the liquid volume with the CT method. *In vitro* SDH concentrations correlated in a linear manner with signal intensity until 200 mg/ml (Fig. 1A). At higher concentrations the signal intensity tended to plateau but volume detection remained accurate. To test if the CT method can detect volume changes, we measured different liquid volumes containing equal amounts of SDH with the CT method. Volume changes showed an excellent linear correlation without a loss in signal intensity *in vitro* (Fig. 1B). To exclude that pH such as a low pH in the stomach affected SDH quantification, we measured SDH signal intensity at different pH values *in vitro*. pH did not affect signal intensity down to pH 3 (Fig. 1C). However, at more acid pH SDH precipitated.

In vivo quantification of SDH concentration and liquid volume by micro-CT

To test SDH quantification and volume detection *in vivo* we performed similar experiments as described above for *in vitro* measurments. Briefly, 4 h fasted rats were anesthetized and different concentrations or volumes of SDH were applied orally. Animals were immediately imaged by CT and stomach total volume and signal intensity assessed (Fig. 2A). Image resolution enables even to distinguish the anatomical difference of the stomach wall between the antrum and the body. Applied SDH concentration and stomach intensity correlated linearly $R^2 = 0.99$ (Fig. 2B). Stomach volume detection, which is based on the presence of SDH, is accurate when the stomach SDH content is larger than 100 mg (Fig. 2B). When different volumes containing the same amount of SDH were given by gavage, the applied volumes correlated linearly with the measured stomach volume $R^2 = 0.96$ (Fig. 2C). Importantly, the stomach was not completely empty after 4 h fasting and there was a residual

gastric volume of 0.81 cm^3 – here termed non-administered stomach volume. Hence, *in vitro* and *in vivo* SDH concentration and volume changes were accurately detected by the applied CT method. Therefore all following experiments were conducted with a fixed oral administration volume of 2 ml and a SDH concentration of 200 mg/ml i.e. 400 mg/animal.

mCT validation against the classical phenol red method

Next we validated the CT method by comparing it to classical lethal methods (12). Gastric emptying was inhibited in half of the animals by pre-treating them with CCK (4 µg/kg IP) and after 20 min the extent of gastric emptying was quantified by different methods. Stomach wet weight, total stomach volume, phenol red and SDH content were increased in the CCK treated animals compared to saline control (Fig. 3). Importantly, the methods measuring stomach content showed similar relative increases after CCK administration.

Stomach function under anesthesia

A major advantage of *in vivo* imaging is the capability to assess the same phenomena in the same animal repeatedly. CT imaging requires an immobilized animal, but certain anesthetics such as isoflurane were shown to delay gastric emptying (23). Here, we compared stomach SDH content, total volume and non-administered volume in animals treated with a light anesthetic (Zoletil) to non-anesthetized animals. All animals received an oral SDH gavage. After application the non-anesthetized animals were returned to their home cage and not exposed to anesthesia. After a specific time window one CT image was acquired of these animals (here, termed endpoint anesthesia). Zoletil treated animals were under constant anesthesia, hence sequential imaging of the same animal over time was possible. Zoletil was administered IP and SC to test if the exposure of the GI tract to the anesthetic altered gastric function. Under all conditions, stomach SDH content, total volume and the non-administered volume decreased similar over time (Fig. 4). Noteworthy, the endpoint anesthesia group had a

higher variability due to intra-individual differences in gastric function. This demonstrates that Zoletil anesthetized animals have similar gastric function as non-anesthetized animals and therefor bear the advantage to allow repeated measures of stomach content over time in the same animal. Importantly the gastric emptying curve was similar to the exponentially emptying of non-caloric meals observed by MRI in humans or phenolred in rats (12, 20). This indicates that SDH did not interfere with normal gastric emptying.

Pharmacological modulation of gastric emptying and secretion by CCK and histamine

To further validate our method, we pharmacologically altered gastric function by applying CCK or histamine. Their impact was monitored at a high temporal resolution. CCK was previously reported to delay gastric emptying and devazepide is a known CCK antagonist (12, 18). Indeed, CCK (4 µg/kg IP) completely inhibited stomach SDH release for 20 min (Fig. 5A). Additionally, CCK induced an increase of the stomach total and non-administered volume presumably due to enhanced secretion (Fig. 5B & 5C). Devazepide (1 mg/kg IP) completely antagonized the effect of CCK, but alone did not differ from vehicle control. Next, we pharmacologically induced gastric secretion by administering histamine (5 mg/kg IP) (5). Histamine injection delayed SDH and total stomach volume decreased (Fig. 6A & B). Importantly, non-administered volume increased after 20 min indicating gastric secretion (Fig. 6C). Together, these results show that the CT method can detect the expected pharmacological effects of CCK and histamine on gastric emptying and secretion with an excellent temporal resolution.

Oral glucose empties at a rate of 24-31 cal/min

Finally, we evaluated if physiologically induced meal-born signals can modulate gastric function. Most prominently orally applied glucose solutions were shown previously to calibrate gastric emptying to a fix rate of 30-45 cal/min in rats (14). To test these phenomena,

we orally administered 0.2 and 0.4 g/ml glucose, respectively, in combination with the contrast agent and monitored gastric function by CT over time. Indeed, glucose delayed the release of SDH content and induced an increase of total and non-administered stomach volume (Fig. 7A, 7B & 7C). When plotting the energy (in kJ) emptied from the stomach over time, a linear regression could be fitted which suggests a linear gastric emptying rate of 24-31 cal/min (Fig. 7D; not statistically different). This highlights that our CT method can detect and reproduce the expected effect of oral glucose on gastric emptying.

Discussion

Gastric emptying and secretion are two key functions of the stomach. The current methodology does not allow the assessment of both simultaneously in small mammals like rats *in vivo* (7, 22). Here, we demonstrate that based on CT, it is possible to accurately measure the volume and concentration of SDH, a liquid contrast agent, *in vitro* but also in the stomach of living animals (Fig 1 & 2). Gastric emptying therefore can be quantified by measuring changes of the absolute SDH content in the stomach over time, and gastric secretion can be calculated based on stomach SDH concentration and volume. Changes of the total stomach volume depend on the volumes emptied, secreted or ingested within the specified time frame. The ingested volume can be neglected when animals have no access to food or drinking water during the study. The emptied volume can be calculated based on the changes in the total SDH content. Therefore, the remaining volume reflects the volume which has not been administered and which can therefore be approximated as gastric secretion. This assumes proper and rapid mixing of the contrast agent with the secreted volume, emphasizing the importance of a liquid nature of a contrast agent. Similar reasons to assess gastric emptying and secretion have also been made in respect to human MRI data sets (20). Furthermore, we show a good correlation between classic lethal methods and our CT approach as validation (Fig. 3). Additionally image resolution is excellent enabling to distinguish anatomical stomach features.

Non-lethal *in vivo* methods provide the major advantage to measure a function in the same animal over time. Here, we validated the use of a light anesthetic that does not inhibit gastric emptying and can be used for high temporal resolution studies (Fig 4). These technical advantages were applied to validate the method with pharmacological modulators of GI function and physiologically induced meal born signals. Among them are several GI hormones which contribute to proper GI function. CCK, for example, stimulates the

relaxation of the proximal stomach, increases its reservoir function and inhibits gastric emptying. This has been documented as delayed phenol red release upon CCK treatment that is antagonized by devazepide (12, 18). Our data confirmed this effect with high temporal resolution (Fig. 5). Interestingly, CCK inhibited stomach SDH release completely for 20 minutes which might lead to the accumulation of basal gastric acid secretion. This would also explain the observed increase of the total stomach volume and non-administered volume. Alternatively, CCK might induce gastric secretion based on its structural relationship to gastrin (3). Histamine stimulates gastric secretion but it can also delay gastric emptying by interacting with the H1-receptor (5, 6). Our data clearly support both effects and confirm its action to inhibit gastric emptying and to increase gastric secretion (Fig. 6). Endocrine and neuronal signals are physiologically induced by the ingested meal itself. Most prominently gastric emptying is described to be modulated mainly by the caloric equivalent of the ingested meal. Glucose solutions were shown to empty at a fix rate of 2-2.5 kcal/min in humans and at a rate of 30-45 cal/min in rats (9, 14). We confirmed this finding and additionally showed an increase in gastric secretion upon glucose ingestion possibly due to an insulin effect (Fig. 7).

Taken together, we developed and validated a method to simultaneously measure gastric secretion and emptying in rats *in vivo* based on non-invasive CT. This enables paired experiments and reduces future harmful animal experiments. Furthermore, we tested a light anesthetic strategy to increase temporal resolution of gastric experiments. As new CT scanners are getting broadly available, this method should be exploited for future efforts that may aim of measuring intestinal or colonal transit motility and volume.

Acknowledgements

We would like to thank Brigitte Herzog and Simone M. R. Camargo for their great support throughout the study, the Zurich Integrative Rodent Physiology Facility and Petra Seebeck for the use of the CT and the Zurich Center for Microscopy and Imaging Analysis for the use of their computers. Additionally we thank the Group of F.V. and T.L. for encouraging discussions.

Grants

This work was supported by Zurich Center of Integrative Human Physiology (ZHIP), the Swiss NSF grant 31-130471/1 to FV and 31003A-138 246 to TAL

Disclosures

No conflicts of interest, financial or others are declared by all authors.

Author contributions

All authors contributed to study design and data interpretation. J.J. performed all experiments and wrote the paper with the help of T.A.L. and F.V. All authors approved the final version of the manuscript and all authors qualifying for authorship are listed.

References

1. **Adelson DW, Million M, Kanamoto K, Palanca T, Taché Y.** Coordinated gastric and sphincter motility evoked by intravenous CCK-8 as monitored by ultrasonomicrometry in rats. *Am J Physiol Gastrointest Liver Physiol* **286**:G321-G332; 2004.

2. **Bennink RJ, De Jonge WJ, Symonds EL, van den Wijngaard RM, Spijkerboer AL, Benninga MA, Boeckxstaens GE.** Validation of Gastric-Emptying Scintigraphy of Solids and Liquids in Mice Using Dedicated Animal Pinhole Scintigraphy. *J Nucl Med* **44**:1099–1104, 2003.

3. **Camilleri M.** Integrated upper gastrointestinal response to food intake. *Gastroenterology* **131**:640–658.

4. **Creedon CT, Verhulst PJ, Choi KM, Mason JE, Linden DR, Szurszewski JH, Gibbons SJ, Farrugia G.** Assessment of Gastric Emptying in Non-obese Diabetic Mice Using a [13C]-octanoic Acid Breath Test. *J Vis Exp*:e50301, 2013.

5. **Furutani K, Aihara T, Nakamura E, Tanaka S, Ichikawa A, Ohtsu H, Okabe S.** Crucial Role of Histamine for Regulation of Gastric Acid Secretion Ascertained by Histidine Decarboxylase-Knockout Mice. *J Pharmacol Exp Ther* **307**:331–338, 2003.

6. **Bertaccini G, Scarpignato C.** Histamine H2-antagonists modify gastric emptying in the rat. *Br J Pharmacol* **77**:443–448, 1982.

7. **Ghosh T, Lewis DI, Axon AT, Everett SM.** Review article: methods of measuring gastric acid secretion. *Aliment Pharmacol Ther* **33**:768–781, 2011.

8. **Holdsworth DW, Thornton MM.** Micro-CT in small animal and specimen imaging. *Trends Biotechnol* **20**:S34, 2002.

9. **Hunt JN, Smith JL, Jiang CL.** Effect of meal volume and energy density on the gastric emptying of carbohydrates. *Gastroenterology* **89**:1326–1330, 1985.

10. **Lebovitz HE.** Adjunct therapy for type 1 diabetes mellitus. *Nat Rev Endocrinol* **6**:326–334, 2010.

11. **Liao D, Zhao J, Gregersen H.** Three-dimensional geometry analysis of the stomach in type II diabetic GK rats. *Diabetes Res Clin Pract* **71**:1–13, 2006.

12. **Mangel AW, Koegel A.** Effects of peptides on gastric emptying. *Am J Physiol* **246**:G342-5, 1984.

13. **Mard SA, Gharib Naseri MK, Badavi M.** Gastric secretions affected by esophageal distention in the rat. *J Gastroenterol* **44**:132–138, 2009.

14. **McCann MJ, Stricker EM.** Gastric emptying of glucose loads in rats: effects of insulin-induced hypoglycemia. *Am J Physiol* **251**:R609-13, 1986.

15. **McColl KEL, El-Omar E, Gillen D.** Interactions between H. pylori infection, gastric acid secretion and anti-secretory therapy. *Br Med Bull* **54**:121–138, 1998.

16. **Medhus AW, Lofthus CM, Bredesen J, Husebye E.** Gastric emptying: the validity of the paracetamol absorption test adjusted for individual pharmacokinetics. *Neurogastroenterol Motil* **13**:179–185, 2001.

17. **Rayner CK, Horowitz M.** New management approaches for gastroparesis. *Nat Clin Pract Gastroenterol Hepatol* **2**:454–462, 2005.

18. **Reidelberger RD, Kelsey L, Heimann D, Hulce M.** Effects of peripheral CCK receptor blockade on gastric emptying in rats. *Am J Physiol Regul Integr Comp Physiol* **284**:R66-75, 2003.

19. **Roda A, Mezzanotte L, Aldini R, Michelini E, Cevenini L.** A new gastric-emptying mouse model based on in vivo non-invasive bioluminescence imaging. *Neurogastroenterol Motil* **22**:1117–e288, 2010.

20. **Sauter M., Curcic J, Menne D, Goetze O, Fried M, Schwizer W, Steingoetter A.** Measuring the interaction of meal and gastric secretion: a combined quantitative magnetic resonance imaging and pharmacokinetic modeling approach. *Neurogastroenterol Motil* **24**:632–e273, 2012.

21. **Schubert ML, Peura DA.** Control of Gastric Acid Secretion in Health and Disease. *Gastroenterology* **134**:1842–1860, 2008.

22. **Szarka LA, Camilleri M.** Methods for measurement of gastric motility. *Am J Physiol Gastrointest Liver Physiol* **296**:G461-G475, 2009.

23. **Torjman MC, Joseph JI, Munsick C, Morishita M, Grunwald Z.** Effects of Isoflurane on gastrointestinal motility after brief exposure in rats. *Int J Pharm* **294**:65–71, 2005.

24. **Tsurugizawa T, Uematsu A, Nakamura E, Hasumura M, Hirota M, Kondoh T, Uneyama H, Torii K.** Mechanisms of neural response to gastrointestinal nutritive stimuli: the gut-brain axis. *Gastroenterology* **137**:262–273, 2009.

Figures

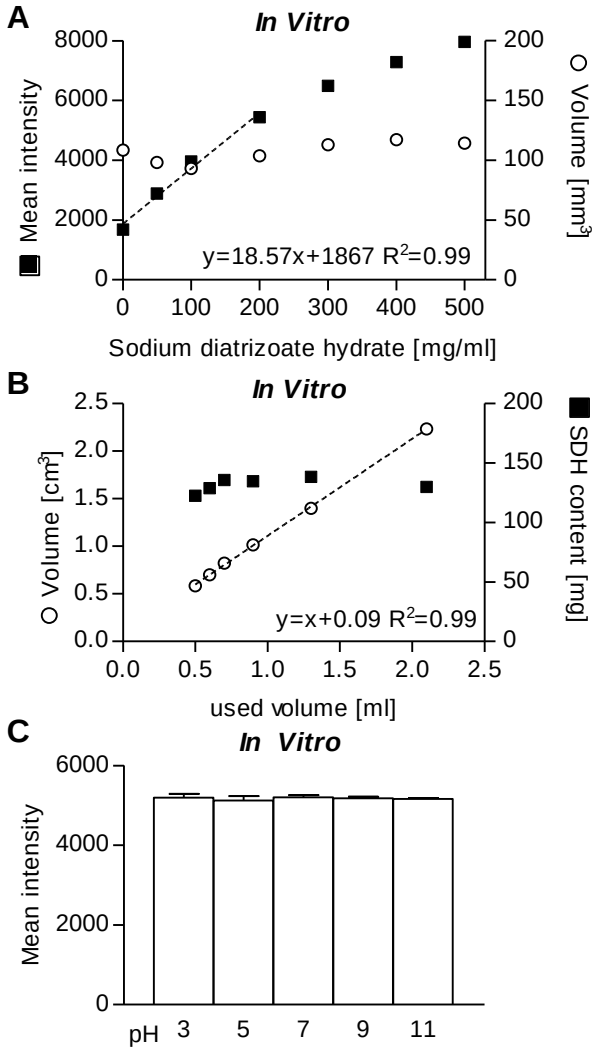


Figure 1 – In vitro calibration of SDH concentration and solution volume measurement by micro CT. (A) CT measurement of *in vitro* signal intensity and volume of solutions with different SDH concentrations but equal volume (120 µl). Linear regression accounts for all data until 200 mg/ml. (B) *In vitro* signal intensity and volume of solutions with different volumes but equal SDH content (125 mg). (C) *In vitro* signal intensity of solutions with equal volume and SDH content but at different pH. Mean ± SEM, n = 3.

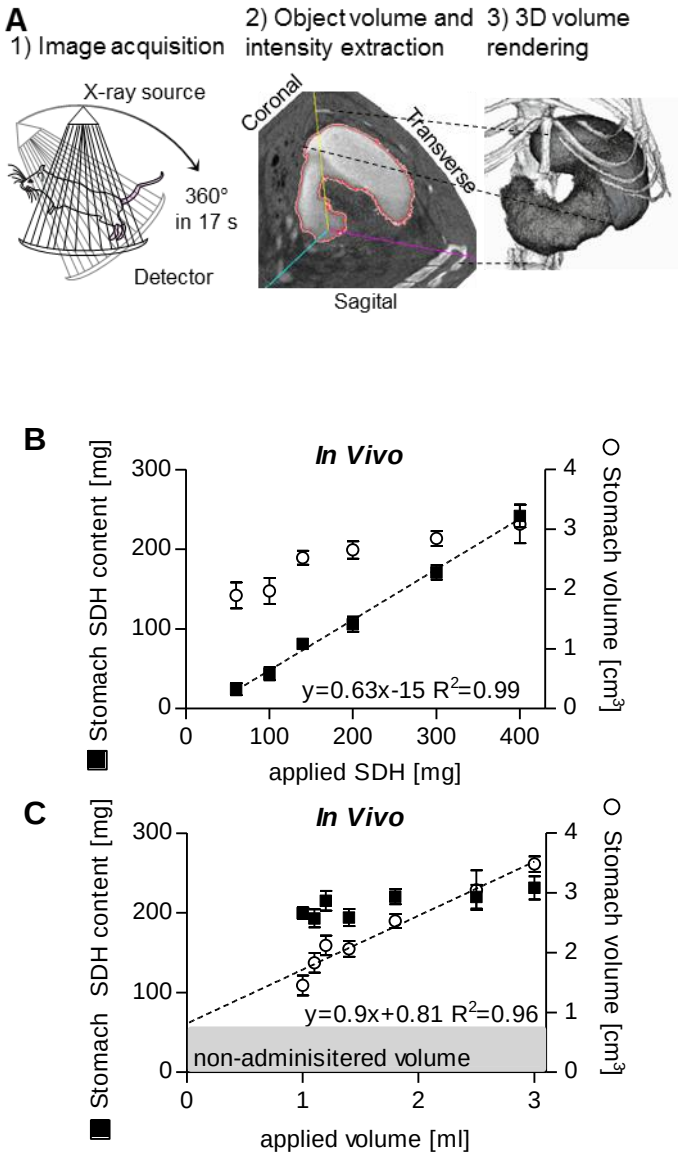


Figure 2 – In vivo calibration of SDH concentration and solution volume measurement by micro CT. (A) Workflow of *in vivo* experiments starting with image acquisition. Next, the stomach is semi-automatically segmented (red line) and objects volume and mean intensity extracted. This object is subject to 3D volume rendering for image presentation. (B) *In vivo* signal intensity and volume obtained from the stomach immediately after oral application of different SDH concentrations with equal gavage volumes (2 ml). (C) *In vivo* signal intensity and volume recorded from the stomach immediately after oral application of different volumes with equal SDH content (200 mg). Mean ± SEM, n = 4-5.

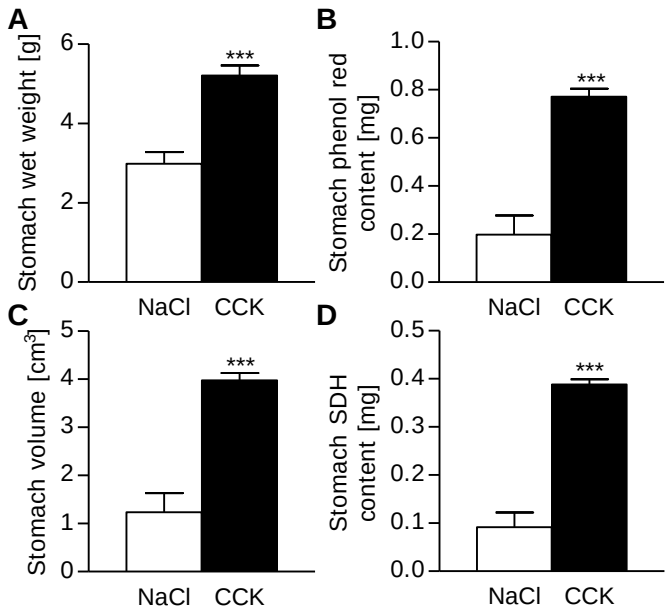


Figure 3 – Comparison of micro CT and phenol red method. Animals were treated with CCK (IP, 4 µg/kg) and received a 2 ml oral SDH (200 mg/ml) and phenol red (0.75 mg/ml) application. Stomach volume and SDH content was measured by CT 20 min post-application. The same stomachs were then excised and their wet weight and phenol red content assessed. Mean ± SEM, n = 5, unpaired two-tailed student t-test, ***$p<0.001$.

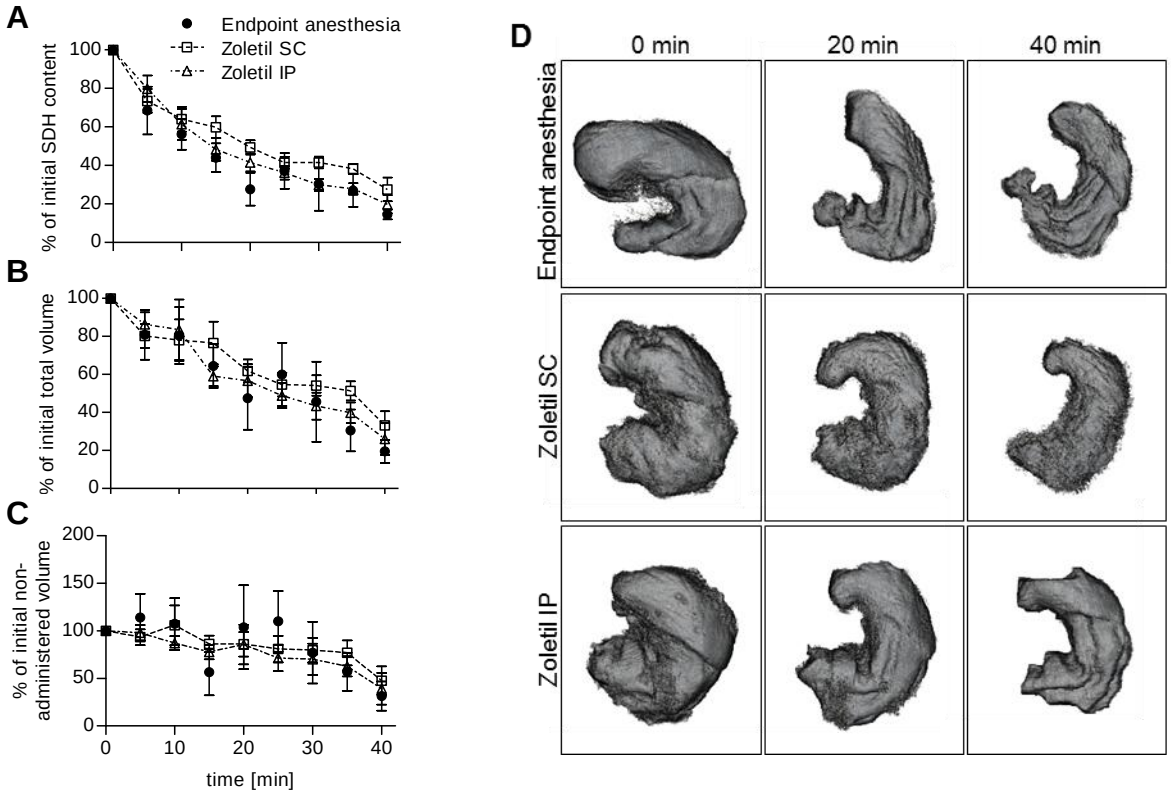


*Figure 4 – Comparison of stomach function under continuous Zoletil and endpoint isoflurane anesthesia.*The effect of different anesthetics on stomach SDH content (A), volume (B) and secretion (C) was analyzed after oral SDH application. The endpoint anesthesia group is composed of animals just anesthetized after a specific time point to acquire one image (n = 6 / time point; total = 54 animals). In contrast, the Zoletil groups are the same animals imaged every 5 minutes as indicated by the connecting lines (n = 6). (D) Representative 3D volume renderings of stomachs are shown. Mean ± SEM, no statistical significant differences were found.

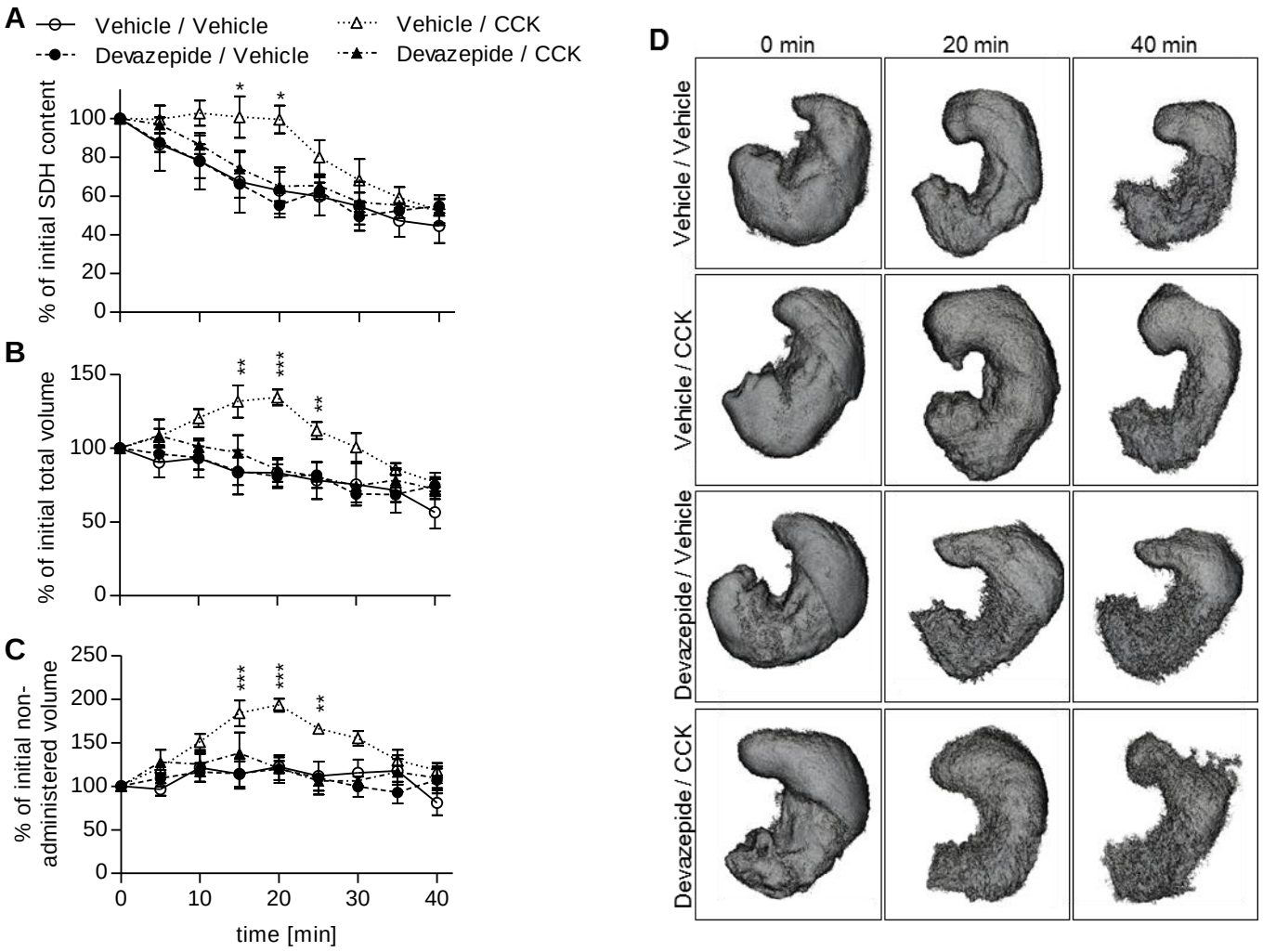


Figure 5 – Effect of CCK application on stomach function measured by micro CT. Animals were treated with CCK (IP, 4 µg/kg) and the effect of CCK antagonized applying devazepide (IP, 1 mg/kg). Stomach SDH content (A), volume (B) and secretion (C) was recorded over time in the same animal after oral SDH application. (D) Representative 3D volume renderings of stomachs are shown. Mean ± SEM, n = 5, unpaired two-way ANOVA, Bonferroni post test; *p < 0.05, **p < 0.01, ***p < 0.001.

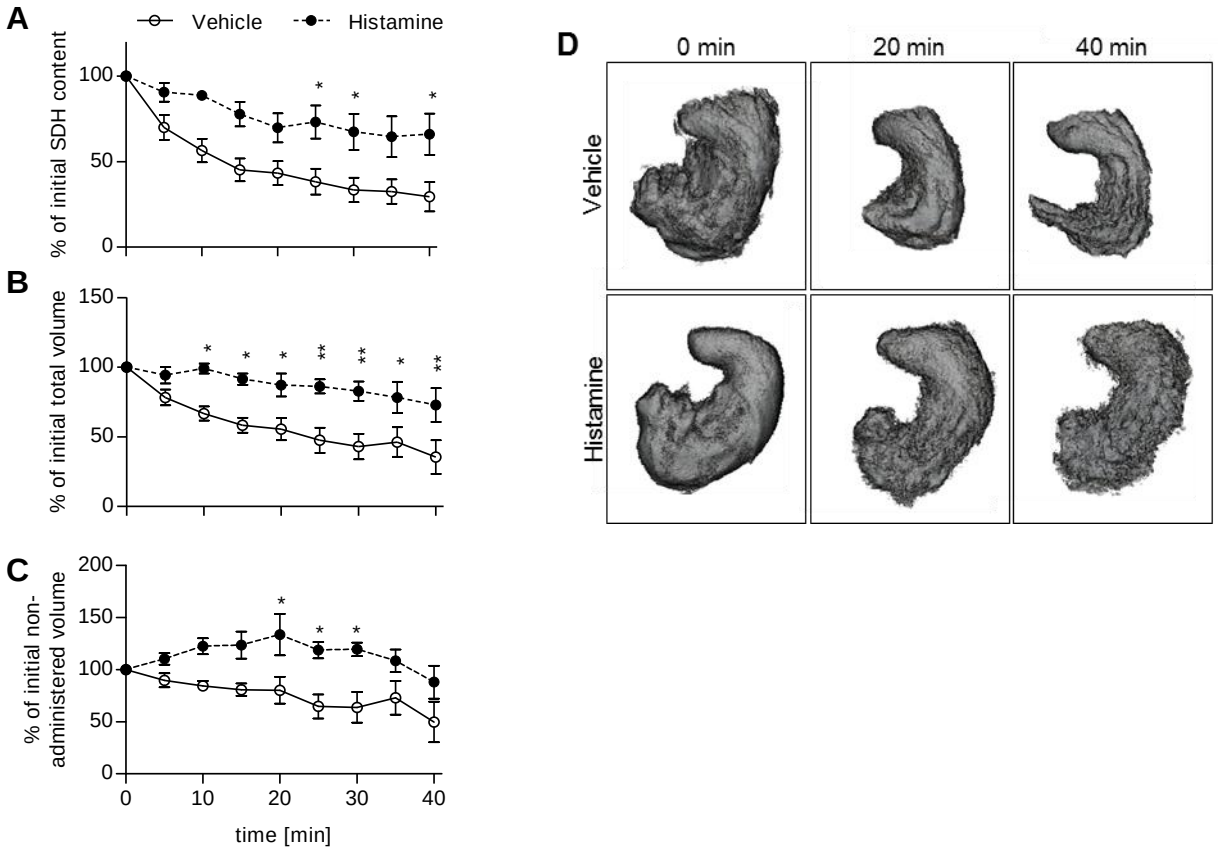


Figure 6 – Effect of histamine application on stomach function measured by micro CT.

Animals were treated with histamine (IP, 5 mg/kg). Stomach SDH content (A), volume (B) and secretion (C) were recorded over time in the same animal after oral SDH application. (D) Representative 3D volume renderings of stomachs are shown. Mean ± SEM, n = 4-5, unpaired two-way ANOVA, Bonferroni post test; *$p < 0.05$, **$p < 0.01$.

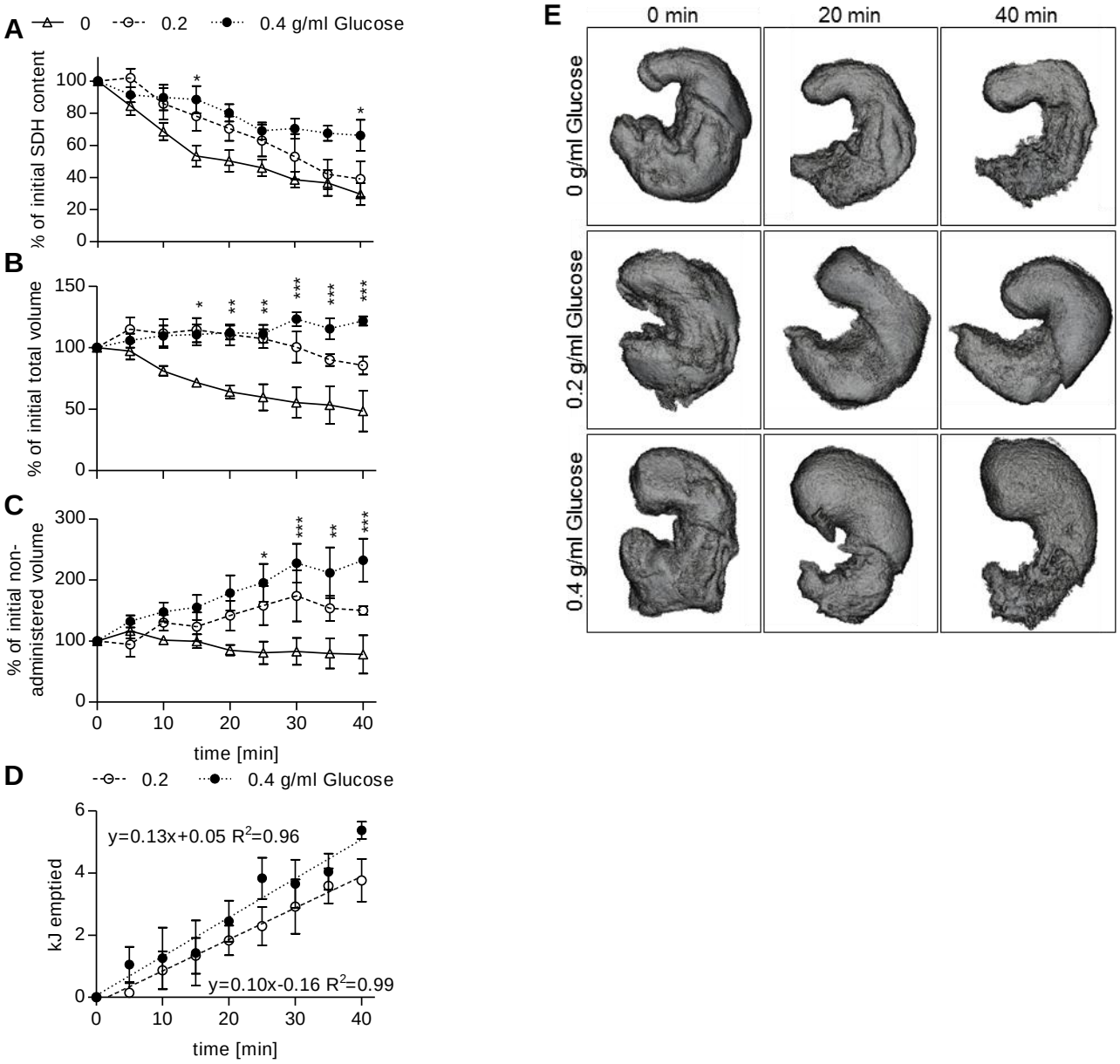


Figure 7 – Effect of glucose application on stomach function measured by micro CT. Animals received different oral glucose solutions including SDH. Stomach SDH content (A), volume (B) and secretion (C) was recorded over time in the same animal after application. kJ emptied form the stomach calculated based on the emptied SDH content are plotted over time in panel D and a linear regression fitted. (E) Representative 3D volume renderings of stomachs are shown. Mean ± SEM, n = 4, unpaired two-way ANOVA, Bonferroni post test; *p < 0.05, **p < 0.01, ***p < 0.001

Equations

SDH concentration *in vitro*$_{t = x\ min}$ [mg / ml] = (mean intensity – 1867) / 18.57 (see Fig. 1A)

SDH content *in vivo*$_{t = x\ min}$ [mg] = {(SDH concentration *in vitro*$_{t = x\ min}$ [mg / ml] * total volume$_{t = x\ min}$ [ml]) + 15} / 0.063 (see Fig. 2A)

Gastric emptying: <u>% of initial SDH content</u> = SDH content *in vivo*$_{t = x\ min}$ [mg] / SDH content *in vivo*$_{t = 0\ min}$ [mg] * 100

<u>% of initial total volume</u> = total volume$_{t = x\ min}$ [ml] / total volume$_{t = 0\ min}$ [ml] * 100

Gastric secretion: <u>% of initial non-administered volume</u> = Non-administered volume $_{t = x\ min}$ [ml] / Non-administered volume $_{t = 0\ min}$ [ml] * 100

Non-administered volume $_{t = x\ min}$ [ml] = total volume$_{t = x\ min}$ [ml] – SDH volume$_{t = x\ min}$ [ml]

SDH volume$_{t = x\ min}$ = 2 ml * SDH content *in vivo*$_{t = x\ min}$ [mg] / 400 mg (we initially apply 2 ml of SDH 200 mg/ml)

6. Original Research Article: “L-Lysine dose dependently delays gastric emptying and increases intestinal fluid volume in humans and rats”

This section contains an original research article, which is under internal review of the co-auhors. The article will be submited to the Journal of Physiology until the end of September.

My contribution to this paper includes aspects in the study design, data interpretation and preliminary experiments in humans. I performed all animal experiments. C. Baruffol performed all human experiments, together we wrote the paper and therefore we share 1st authorship. A. Steingötter, T. Lutz and F. Verrey supervised all aspects of the project.

Title: L-lysine dose dependently delays gastric emptying and increases intestinal fluid volume in humans and rats

Authors: Carolin Baruffol[1,*] / Josua Jordi[2,3,4,*], Simone Camargo[3,4], Tijana Radovic[1], Brigitte Herzog[3,4], Michael Fried[1,4], Werner Schwizer[1,4], François Verrey[3,4] , Thomas A. Lutz[2,4] , Andreas Steingoetter[1]

Affiliations: [1]Division of Gastroenterology and Hepatology, University of Zurich, Switzerland; [2]Institute of Veterinary Physiology, University of Zurich, Switzerland; [3]Institute of Physiology, University of Zurich, Switzerland; [4]Zurich Center for Integrative Human Physiology (ZIHP), University of Zurich, Switzerland; *These authors contributed equally to this work.

Additional information:

A) Running Title: The impact of L-lysine on gastrointestinal function

B) Keywords: L-lysine, gastrointestinal function, MRI

C) Total words in the paper excluding references and figure legends:

D) Corresponding Author: Andreas Steingoetter

Division of Gastroenterology and Hepatology

University of Zurich,

Raemistrasse 100, CH-8091 Zurich, Switzerland

Phone: +41 44 255 56 84 / Fax: +41 44 255 45

steingoettter@biomed.ee.ethz.ch

E) Table of contents: Integrative

Abstract:

Novel sensory inputs for the control of food intake and gastrointestinal (GI) function are of increasing interest due to the rapid increase in nutrition related diseases. The essential amino acid L-lysine, along with L-arginine and L-glutamic acid, was demonstrated to have a selective impact on food intake and GI processing in rats thus indicating a potential novel direct sensory input to assess dietary protein content and quality. The aim of this study was to assess the translational aspects of this finding and to investigate the dose-dependent effect of L-lysine on human and rat GI function. L-lysine doses from 0-800 mg in rats and 0.5-7.5 g in humans were analyzed for their effect on gastric emptying and gastrointestinal secretion. Human GI function was non-invasively assessed using magnetic resonance imaging (MRI) whereas rat data was acquired using standard lethal measurement methods. L-lysine dose-dependently delayed gastric emptying and stimulated gastrointestinal secretion in rats as reflected by increased gastric wet weight and residual phenol red content (0.4g and 3% per 0.1g L-lysine, $p<0.001$). The dose-dependent delay in gastric emptying was confirmed in humans with an increase in half-time of gastric emptying of 4 min/g L-lysine, $p<0.01$. Moreover, a dose-dependent increase in intestinal fluid accumulation was observed (0.4 ml/min/g L-lysine, $p<0.0001$). No effect on blood gas parameters or visceral sensations was detected. This translational study demonstrates comparable dose-dependent effects of intragastric L-lysine on GI function in humans and rats. This conserved chemospecificity supports a broader role for individual amino acids in the control of gastrointestinal motility and secretion *in vivo*.

Abbreviation List:

CCK, Cholecystokinin; GI, gastrointestinal; MRI, Magnetic Resonance Imaging;

LMH – L-lysine monohydrate

Introduction:

The worldwide rapid increase in nutrition related diseases stimulated efforts to understand how a meal impacts on various physiological processes including gastrointestinal (GI) function. Upon food intake, GI function adapts quickly to the ingested meal presumably to control efficient digestion and absorption of the vital nutrients. This dynamic plasticity is regulated by mechanisms responding to properties specific to the ingested meal among them are caloric content, volume and consistency. This multiple signalling input is integrated to some extent by the autonomous nervous system but also by the central nervous system via vagal and endocrinal signals to control proper GI function (Camilleri, 2006).

The macronutrient composition of a meal is historically not considered to have a major impact on GI function (Cummings & Overduin, 2007; Boron & Boulpaep, 2012). This is in contrast to newly emerging concepts demonstrating macronutrient specific control of different vagal and endocrinal signals. For instance, specific individual amino acids induce vagal afferent firing or stimulate the release of different GI hormone *ex vivo* (Tanaka *et al.*, 1990; Niijima, 1991; Niijima & Meguid, 1995; Niijima, 2000; Torii & Niijima, 2001). The *in vivo* relevance of these findings in context of GI function control can only be extrapolated from studies showing that high protein diets delayed gastric emptying to a larger extends than their isocaloric control in both humans and rats (Blom *et al.*, 2006; Faipoux *et al.*, 2006). Controversially others could not confirm these observations including us (Goetze *et al.*, 2007). As high protein diets are composed of different individual amino acids and thereby considerably vary between laboratories, this might obscure amino acid specific effects. So far, no study systematically tested the impact of all the 20 individual amino acids on GI function in humans or rats. Therefore the selection of a candidate amino acid was based on a recent study in rats revealing that L-arginine, L-lysine and L-glutamic acid induced the most potent anorectic effect compared to all other proteogenic amino acids (Jordi *et al.*, 2013). Within this

study L-lysine was shown to delay solid gastric emptying, induce gastric secretion and increase intestinal motility thereby revealing an amino acid specific impact on GI function *in vivo*. Still several aspects remained elusive. Neither the dose-dependent effect nor the temporal dynamics of L-lysine were resolved and which both are relevant for the characterization of the pharmacokinetic effect of L-lysine. Most important it remained unclear if humans show a similar gastrointestinal response to a gastric load of L-lysine. This is relevant, as humans and rodents significantly differ in GI anatomy and physiology even though from an ingestive perspective both are omnivores. For instance, rats eat 10-20 % of their body weight equivalent as food every day, whereas healthy humans at best 2 %. Moreover, rats do not have a gallbladder and lack the vomiting reflex.

The aim of this study was to identify the dose-dependent effect of L-lysine on human and rat GI function after intragastic infusion or gavage, respectively. Animal experiments were restricted to the 30 min time point due to lethal methods. GI function was analysed by measurements of wet weight and residual phenol red content. In humans, magnetic resonance imaging (MRI) and blood sampling was applied to repeatedly assess gastric meal, secretion and intestinal fluid content volume as well as blood gas parameters every 15 min for over 90 min. Human experiments were performed within a randomized, double-blind, three-armed, cross-over study design. The study revealed a conserved impact of L-lysine on GI function in humans and rats.

Methods:

Ethical approval

All procedures for rat handling and experimental interventions were according to the Swiss Animal Welfare laws and approved by the Kantonale Veterinäramt Zürich. The human study was performed between April and September 2012 in a single centre, randomized, double-blind, 3-armed, unbalanced, cross-over study design and in accordance with the Declaration of Helsinki, Good Clinical Practice. It was registered at ClinicalTrial.gov with identifier NCT01212614. The local Ethics Committee approved the study and all volunteers gave written informed consent.

Animal care

30 male Wistar rats (Janvier, Saint Berthevin Cedex, France) were grouped housed (room temperature 21 ± 1° C, artificial 12/12 h light dark cycle, water ad libitum, rat chow ad libitum - 3433 Kliba Nafag, Kaiseraugst, Switzerland).

Animal dose preparation

0, 25, 50, 100, 200 and 400 mg/ml L-lysine (Sigma-Aldrich, Buchs, Switzerland) was solubilized in tap-water and 80, 75, 70, 60 and 40 mg/ml NaCl (Sigma) proportionally added to correct for molarity differences. pH was adjusted to 7.2 using HCl (Merck, Zug, Switzerland). The applied volume was 2 ml for each of these solutions supplemented with 1.5 mg phenol red (Sigma).

Animal experiment design

Animals were food deprived for 4 h prior oral L-lysine administration to ensure similar residual stomach content. Rats received intragastric application of 2 ml of the respective L-lysine dose and were returned to their home cage, where they did not have access to food or

water. 30 min post application animals were euthanized using pentobarbital (IP, 100 mg/kg, Kantonsapotheke Zürich, Switzerland) in combination with isoflurane (5 %) for quicker induction. The GI tract was excised and segmented into stomach, small intestine, cecum and colon based on anatomical landmarks using clamps to limit liquid loss. The wet weight of each segment was immediately measured. Phenol red was extracted from each segment and quantified by optical spectroscopy at 560 nm wavelength as described previously (Jordi *et al.*, 2013).

Animal Statistics

Rats were randomly allocated into the six treatment groups. Application order was randomized and statistical analysis performed as described for the human data set below.

Human Subjects

A total of six healthy subjects aged between 18-50 years (four male, two female) were recruited via a public advertisement. Prior study inclusion, all subjects underwent a clinical assessment and were tested for negative *helicobacter pylori*. The following exclusion criteria were applied: BMI (kg/m^2) <19 or >24, no history of systemic and psychiatric disease especially GI disease or surgery (excludes appendectomy, hernia repair and anorectal disorders), lysineuria, galactosaemia, medication intake of any kind, drug or alcohol abuse, presence of metallic implants, devices or metallic foreign bodies, claustrophobia, pregnancy and lactation, known allergy or intolerance against locus bean gum, L-lysine, Gadolinium (DOTAREM®), Methylparaben (E218), Fructose, Gluten, Galactose and also allergic reaction after prior injection of Lidocain (e.g. at the dentist). To assure an approximately similar concentration of L-lysine per kg of body weight the included subjects had similar body mass indices (22 ± 1 kg/m^2).

Human Test Meal

L-lysine was administered as L-Lysine monohydrate (LMH, Fagron GmbH & Co.KG, Barsbuettel, Germany) at doses of 0.5, 1.2, 3.0 and 7.5 g in four different 300 ml test meals. The ingredients and properties of the four LMH test meals that had negligible caloric content (<25 kcal) are listed in table 1. NaCl was proportionally added to each test meal to correct for molarity differences. All meals were prepared directly before each study session, heated to 37 °C and infused within 1-2 min while volunteers were lying in the MRI scanner.

Human measurement protocol

All six subjects underwent three study sessions of 2.5 hours including subject preparation and repeated MRI and venous blood measurements. Sessions were performed on three different days separated by minimum 5 and maximum 21 days. Subjects were asked to refrain from special foods (chocolate, banana, pineapple, nuts, tomatoes, eggplant, mirabelle, gooseberry, avocados, plums, currant, melon, kiwis, alcohol, coffee, tea and nicotine) for 2 days prior to each study session. Each session followed the same procedure depicted in Figure 1. Subjects arrived at the MR centre in the morning or at noon after fasting overnight (or at least for 8 h). A nasogastric tube (Freka® Tube CH/FR 12) was placed 50-60 cm from the nares after previous local anaesthesia of the nasal cavity with Lidocain-HCl 2 % (prepared in house) and Vibrocil® (Novartis, Basel, Switzerland) nose gel. A venous vascular cannula (Vasofix®Braunüle®) was placed on the right or left cubital vein for subsequent blood sampling and continuous 0.9 % NaCl infusion. MR image data were acquired with subjects positioned in right decubitus before and directly after test meal infusion and then every 15 min until 90 min. Visceral sensations scores were recorded after each MRI scan and in addition at 105 min after meal infusion. Blood samples were collected before (baseline) and at 10, 25, 40, 55, 70, 85 and 100 after meal infusion. The first blood sample in fasted state and last blood samples at 100 min were taken in sitting position, all the others were obtained while

the subject was lying on the MR patient table. The nasogastric tube was always removed at ~60 min with subjects in sitting position.

Human MRI and visceral sensations scores

Subjects were positioned in right decubitus inside the clinical MRI scanner (1.5 T Achieva, Philips Healthcare, Best, NL). An abdominal phased-array surface coil (SENSE body coil, four elements) was used for image acquisition. MR imaging consisted of three imaging sequences to visualize gastric content volume, gastric secretion, gallbladder volume and intestinal fluid content. The MRI scan parameters for gastric content volume measurement were: balanced steady state free precession sequence, 26 - 34 transverse slices, 6 mm slice thickness, no slice gap, 360 mm field of view, 1.6/3.3 ms echo/repetition time, one breath hold, 16 – 24 sec scan duration (depending on slice number). For assessing gastric secretion, MRI scan parameters were: radial T1-weighted gradient echo sequence with profiles acquired in golden angle view order, 18 coronal slices, 6 mm slice thickness, 0.6 mm slice gap, 360 mm field of view, 1.8/12 ms echo/repetition time, free breathing, 2:24 min scan duration. To visualize gallbladder volume and intestinal fluid content, MRI scan parameters were: T2-weighted turbo spin echo sequence with fat suppressions, 26 coronal slices, 5 mm slice thickness, 1 mm slice gap, 375 mm field of view, 80/1200 ms echo/repetition time, two breath holds, 31 sec scan duration.

After MRI scans, subjects had to report their visceral sensations, i.e. hunger, fullness, nausea, bloating, abdominal cramps and urge to defecate using a self-assessed sensations scale ranging from 0 – 10 (0 – no sensations, 10 – extremely prominent sensations).

Human blood sampling and analysis

For blood gas measurements (pH, bicarbonate, pCO2, chloride, glucose, haematocrit), blood was first collected with a serum vacutainer, then filled within 1-3 minutes in heparinized

capillaries and analysed using the ABL 700 blood gas analyser (Radiometer GmbH, Thalwil). For measurements of albumin and L-lysine blood concentration, Li-heparin vacutainer and EDTA-vacutainer were used, respectively. Albumin was measured to assess a potential shift in fluid from intravasal to Intraintestinal. Blood samples were directly centrifuged and ~ 1 ml of plasma was pipetted for storage at -20 °C for L-lysine and 8 °C for albumin. 50 µl of each L-lysine plasma sample was mixed with 50 µl of 10 % SSA/NVal solution and centrifuged at 15'000 rpm for 5 min. 60 µl borate/NaOH buffer and 20 µl reagent (2AMT) was added to 20 µl of the resulting supernatant and 1/100 µl of this mixture was injected on a HPLC column using the cell culture gradient, according to the manufacture's protocol. The plasma for the albumin measurement was processed with a Cobas 8000 modular analyser (c701 module, Roche, Mannheim) subsequent a colour test method (Bromocresol green) with endpoint measure.

Data analysis

MR image analysis was performed by a radiologist in training (TR) and a research assistant with one year of training in analysing abdominal MR image data (CB). Purpose built software tools implemented in MATLAB (The MathWorks, Natick, MA) and IDL (Exelis Visual Information Solutions) were applied for extraction of gastric content volume, gastric secretion, gallbladder volume and intestinal fluid content. Gastric content contours were segmented in each transverse image by a semi-automated algorithm. Respective 3D gastric content volumes were computed from 3D isosurfaces of the contours (Sauter *et al.*, 2012). Gallbladder contours and small intestinal areas excluding blood vessels and visceral tissues were manually contoured in each T2-weighted coronal image slice. To assess intestinal fluid content, a signal threshold was selected to segment the bright luminal fluid content from air and surrounding soft tissue. Intestinal fluid volume was calculated by summing all segmented

pixels. Gastric secretion volume was analysed from reconstructed T1 maps according to a previously established analysis method (Sauter *et al.*, 2012).

MR parameters were smoothed using function *loess* (with smoothing parameter $\alpha = 1$) in program R (R Development Core Team, 2008) and plotted over time and LMH dose. Half-times of gastric emptying (t_{50}) were calculated from smoothed curves by solving the locally smoothed interpolation for 50% using the Newton method (Brent, 1973).

Dose response of t_{50} was analysed by linear mixed model with t_{50} and LMH dose as fixed effects and subject as random effect. Dose response of gallbladder and intestinal fluid volumes was analysed by linear mixed model with volume, time and LMH dose as fixed effects and subject as random effect.

The density functions of individual mean visceral sensations scores were plotted. Since the resulting distributions were dominated by zero values, visceral sensations scores were divided into two categories, i.e. 0 and ≥1, to only test for a dose dependent increase in scores not zero. The dose dependent effect of L-lysine on the binomial visceral sensations scores was analysed using generalised linear models with a binominal link function.

Deltas over baseline (DOB) values were calculated for L-lysine and albumin concentrations, all blood gas parameters and base excess. Baseline was defined as the values detected at fasted state. DOB values were smoothed using function *loess* and plotted over time and LMH dose. Dose response was analysed by linear mixed models with DOB of blood gas parameters, time and LMH dose as fixed effects and subject as random effect. Estimated model parameters are presented as estimate ± standard error. A p-value of < 0.05 was considered significant.

Results:

Animal experiments

To identify a dose dependent impact of L-lysine on rat GI function, we administered different isomolar doses of L-lysine intragastrically and measured GI tract wet weight and phenol red transit 30 min post-application. The doses applied ranged from 0 – 800 mg L-lysine per animal covering the span of daily L-lysine intake in rats. Rat stomach and cecum wet weight dose-dependently increased by 0.4 ± 0.04 g / 0.1 g L-lysine ($p < 0.0001$) and 0.2 ± 0.01 g / 0.1 g L-lysine ($p = 0.03$) respectively (Figure 2a, b). No other GI segment showed a dose dependent change in wet weight. As animals did not have access to water or food these wet weights changes reflect alterations in secretion or absorption. To access GI motility more specifically, we tracked the transit of a dye (phenol red) along the GI segments 30 min post application. By measuring residual phenol red in the stomach, we observed delayed gastric emptying by increased gastric phenol red content of 3 ± 0.8% per 0.1 g L-lysine ($p < 0.001$) and decreased small intestinal phenol red content of also 3 ± 0.4% per 0.1 g L-lysine ($p < 0.0001$, Figure 3a, b). In accordance, also colon phenol red content decreased by 0.03/g L-lysine ($p < 0.001$, Figure 3c). The gastric and intestinal phenol red content plateaued at the two higher L-lysine doses. This data shows that L-lysine dose-dependently delayed gastric emptying and stimulated gastric secretion in rats. Additionally, cecum wet weight increased dose dependently indicating alterations in intestinal, pancreatic or biliary secretion. This data is limited to the 30 min time point thereby potentially obscuring lower dose effects of L-lysine.

Human experiments

To assess the translational aspects of L-lysine impact on GI function, we conducted a MRI based study in healthy human subjects. Briefly, we administered different isomolar doses of

L-lysine nasogastrically and measured the impact on GI function and blood gas parameters every 15 min over a time course of 90 min. The administered doses were between 0.5 g and 7.5 g L-lysine per test meal, which is considered physiological relevant as the daily L-lysine intake varies between 2 to 9 g/day (Flodin, 1997). In one subject, measurements of one dose had to be dismissed due to subject non-compliance of the study protocol (subject PB1_1991, 3 g L-lysine dose). After L-lysine application blood L-lysine concentration increased dose-dependently over time by 0.54 ± 0.1 µM/min/g L-lysine ($p < 0.0001$, Figure 4a, b) demonstrating proper application and absorption. No dose dependent differences were found for albumin and all blood gas parameters. (Figure S1, a-c). At the level of the stomach L-lysine dose dependently delayed gastric emptying by an increase in t_{50} of 4.2 ± 1.4 min/g L-lysine ($p = 0.01$, Figure 5a, b), but no changes in gallbladder volume or gastric secretion were detected based on MRI. In the small intestine, L-lysine dose- and time-dependently increased fluid volume by 0.37 ± 0.1 ml/min/g L-lysine ($p < 0.0001$, Figure 6a, b). This effect and its dynamics were clearly detectable in the MR image data (Figure 7). Defecation was altered after the intake of 7.5 g L-lysine, as 5 of 6 subjects reported self-limiting diarrhoea within one to six hours after L-lysine application. No other side effects or dose dependent changes in self-reported visceral sensations were found during the experiments. Original data of all visceral sensations scores and corresponding density functions are summarized in supplemental Figure S2a, b. These findings confirm the dose-dependent delay in gastric emptying and demonstrate an increase in intestinal liquid content with high temporal resolution in humans.

Discussion:

This study revealed several similar functional responses of the GI tract to L-lysine stimulus in humans and rats. In both species, gastric and small intestinal functions were effectively modulated after intragastric infusion of L-lysine.

L-lysine delayed gastric emptying revealing a conserved amino acid specific effect in rats and humans. This delay did not trigger a temporal delay of L-lysine absorption into the blood stream in humans thereby enabling a post-gastric regulatory mechanism. Gastric emptying is generally accepted to be primarily controlled by the volume and the caloric equivalent of the gastric load (Camilleri, 2006). The negligible caloric content and differences between the lowest and highest L-lysine dose in humans (< 23 kcal) and rats (< 5 kcal), however, suggest a novel caloric independent mechanism for the regulation of gastric emptying. This amino acid specific mechanism for the control of GI function has been recently proposed for L-lysine, L-arginine and L-glutamic acid in rats (Jordi *et al.*, 2013) and is herewith confirmed for humans. Beyond that the rat study showed that intravenous L-arginine and L-lysine did not modulate GI function indicating a potential GI luminal mechanism of action (Jordi *et al.*, 2013). Nevertheless, the underlying regulator mechanism might also involve vagal signaling or a paracrine action of GI hormones. Several *ex vivo* electrophysiological studies revealed amino acid specificity of vagal firing as different amino acids excite or inhibit vagal afferent activity dependent on the exposed vagal branch (Tanaka *et al.*, 1990; Niijima, 1991; Niijima & Meguid, 1995; Niijima, 2000; Torii & Niijima, 2001). Interestingly of all amino acids tested only intragastric L-glutamic acid induced vagal signaling *ex vivo* indicating the necessity for amino acid absorption (Uneyama *et al.*, 2006; Kitamura *et al.*, 2011). Hepatic vagal afferents fire specifically to an L-lysine stimulus and they were shown to mediate the anorectic effect of L-lysine in rats (Torii & Niijima, 2001; Jordi *et al.*, 2013). Apart of neuronal signaling L-lysine might stimulate the release of specific GI hormones. For instance

the release of the gut hormone GLP-1 from primary murine intestinal cells was shown to be induced by L-glutamic acids (Tolhurst *et al.*, 2011). Similar L-phenylalanine and L-tryptophan were reported to stimulate CCK secretion from primary cells (Wang *et al.*, 2011). Hence, this demonstrates remarkable amino acid specificity for the secretory function of endocrine cells *ex vivo*. However, the specific impact of L-lysine on GI hormone release and their relevance for the modulation of GI function *in vivo* is currently unknown. Taken together, L-lysine dose dependently delayed gastric emptying revealing a caloric independent mechanism for the control of GI function also in humans.

Gastric secretion was dose dependently induced in animals but not in humans. Importantly, delayed gastric emptying and secretion had a different dose response curve in rats. This indicates that the here applied L-lysine doses in humans might not be sufficient to extensively stimulate gastric secretion. A formerly more invasive study by Sackler et al. applying repeated extraction of gastric content with subsequent titration reported increased gastric HCl secretion after application of 5 g L-lysine combined with a solid test meal (Sackler & Sophian, 1957). Hence, the test meal composition and the sampling method might have synergized with L-lysine to induce increased gastric secretion. It is understood, that gastric secretion is mainly mediated by parietal cells. Here, it was not assessed if L-lysine stimulated parietal secretion directly or induced Histamine secretion by enterochromaffin cells, but as Histamine not only induces gastric secretion but also delays gastric emptying this might be an alternative mediatory mechanism of the observed L-lysine action (Bertaccini & Scarpignato, 1982; Furutani *et al.*, 2003). The missing effect of L-lysine on gastric secretion in humans was reflected and supported by the absence of any differences in blood gas parameters and base excess. Gastric secretion was previously shown to correlate with base excess in blood, an effect termed alkaline tide (Niv & Fraser, 2002). In sum, L-lysine induced gastric secretion at high doses in rats. A similar effect might be probable in humans; however, its analysis is

hampered by the diarrhea inducing side effect of L-lysine doses ≥ 7 g. The reported side effects of self-limiting diarrhea for the highest Lys dose of 7.5 g in humans were accompanied by an increase in intestinal fluid volume. In both humans and rats, a dose dependent increase in intestinal fluid volume, respectively cecum wet weight, was observed. The origin of this fluid accumulation is currently unclear, but might arise either from increased bile and pancreatic secretion, reduced luminal absorption and/or active intestinal fluid influx. A simple osmotic effect can be excluded, because we applied isomolar L-lysine solutions. Since no dose dependent effect on gallbladder volume was detected in humans, and because rats have no gallbladder the latter two possibilities are currently the more likely reasons for this phenomenon.

In healthy humans, the apparent impact of L-lysine on gastric and small intestinal function did not translate into changes in visceral sensation scores. Hunger and fullness were the only scores to exhibit density functions with modes different from zero. Therefore, a simplified ordinal, i.e. binomial, model analysis had to be applied to test for a dose dependent increase in scores ≥ 1. This finding might not be surprising given the low numbers of study participants and the multitude of determinants of visceral sensitivity (Mulak, 2003) . A larger number of volunteers would be needed to conclude on the effect of L-lysine on human satiation.

In conclusion, this translational study demonstrates comparable dose-dependent effects of intragastric L-lysine on GI function in humans and rats, in particular stomach and small intestinal function. This conserved chemospecificity supports a broader role for individual amino acids in the control of gastrointestinal transport and secretion *in vivo*.

Author contribution:

All authors contributed to study design, data interpretation and manuscript revision. C.B performed all human experiments with the help of T.R. J.J. performed all rat experiments with the help of B.H. J.J and C. B. wrote the paper with the help of F.V., T.A.L and A.S. All aspects of the project were supervised by F.V, T.A.L and A.S. All authors contributed to manuscript editing and approved the final version of the manuscript. All authors qualifying for authorship are listed.

Acknowledgments and disclosure:

All authors declare no competing financial, personal, or professional interest that could be construed to have influenced this paper. We want to thank Jelena Curcic and Sena Kuyumcu for their MR technical and medical support, respectively. JJ and this work were supported by Zurich Center of Integrative Human Physiology (ZHIP). The laboratories of FV and TAL are supported by Swiss NSF grant 31-130471/1 and 31-138246, respectively and the National Centre of Competence in Research (NCCR) Kidney.CH (FV).

References

Bertaccini G & Scarpignato C. (1982). Histamine H2-antagonists modify gastric emptying in the rat. *Br J Pharmacol* 77, 443-448.

Blom WA, Lluch A, Stafleu A, Vinoy S, Holst JJ, Schaafsma G & Hendriks HF. (2006). Effect of a high-protein breakfast on the postprandial ghrelin response. *Am J Clin Nutr* 83, 211-220.

Boron WF & Boulpaep EL. (2012). *Medical physiology*. Saunders Elsevier, Philadelphia.

Brent RP. (1973). *Algorithms for Minimization without Derivatives*. Prentice-Hall, Englewood Cliffs, New Jersey.

Camilleri M. (2006). Integrated upper gastrointestinal response to food intake. *Gastroenterology* 131, 640-658.

Cummings DE & Overduin J. (2007). Gastrointestinal regulation of food intake. *J Clin Invest* 117, 13-23.

Faipoux R, Tome D, Bensaid A, Morens C, Oriol E, Bonnano LM & Fromentin G. (2006). Yeast proteins enhance satiety in rats. *J Nutr* 136, 2350-2356.

Flodin NW. (1997). The metabolic roles, pharmacology, and toxicology of lysine. *Journal of the American College of Nutrition* 16, 7-21.

Furutani K, Aihara T, Nakamura E, Tanaka S, Ichikawa A, Ohtsu H & Okabe S. (2003). Crucial role of histamine for regulation of gastric acid secretion ascertained by histidine decarboxylase-knockout mice. *J Pharmacol Exp Ther* 307, 331-338.

Goetze O, Steingoetter A, Menne D, van der Voort IR, Kwiatek MA, Boesiger P, Weishaupt D, Thumshirn M, Fried M & Schwizer W. (2007). The effect of macronutrients on gastric

volume responses and gastric emptying in humans: A magnetic resonance imaging study. *Am J Physiol Gastrointest Liver Physiol* 292, G11-17.

Jordi J, Herzog B, Camargo SM, Boyle CN, Lutz TA & Verrey F. (2013). Specific Amino Acids Inhibit Food Intake via the Area Postrema or Vagal Afferents. *J Physiol*.

Kitamura A, Sato W, Uneyama H, Torii K & Niijima A. (2011). Effects of intragastric infusion of inosine monophosphate and L: -glutamate on vagal gastric afferent activity and subsequent autonomic reflexes. *J Physiol Sci* 61, 65-71.

Mulak A. (2003). Testing of visceral sensitivity. *J Physiol Pharmacol* 54 Suppl 4, 55-72.

Niijima A. (1991). Effect of umami taste stimulations on vagal efferent activity in the rat. *Brain Res Bull* 27, 393-396.

Niijima A. (2000). Reflex effects of oral, gastrointestinal and hepatoportal glutamate sensors on vagal nerve activity. *J Nutr* 130, 971S-973S.

Niijima A & Meguid MM. (1995). An electrophysiological study on amino acid sensors in the hepato-portal system in the rat. *Obes Res* 3 Suppl 5, 741S-745S.

Niv Y & Fraser GM. (2002). The alkaline tide phenomenon. *J Clin Gastroenterol* 35, 5-8.

R Development Core Team. (2008). R: A language and environment for statistical computing., ed. R Foundation for Statistical Computing V, Austria. http://www.R-project.org.

Sackler AM & Sophian LH. (1957). Gastric secretagogue effect of lysine monohydrochloride. *Science* 126, 255-256.

Sauter M, Curcic J, Menne D, Goetze O, Fried M, Schwizer W & Steingoetter A. (2012). Measuring the interaction of meal and gastric secretion: a combined quantitative magnetic resonance imaging and pharmacokinetic modeling approach. *Neurogastroenterol Motil* 24, 632-638, e272-633.

Tanaka K, Inoue S, Nagase H, Takamura Y & Niijima A. (1990). Amino acid sensors sensitive to alanine and leucine exist in the hepato-portal system in the rat. *J Auton Nerv Syst* 31, 41-46.

Tolhurst G, Zheng Y, Parker HE, Habib AM, Reimann F & Gribble FM. (2011). Glutamine triggers and potentiates glucagon-like peptide-1 secretion by raising cytosolic Ca2+ and cAMP. *Endocrinology* 152, 405-413.

Torii K & Niijima A. (2001). Effect of lysine on afferent activity of the hepatic branch of the vagus nerve in normal and L-lysine-deficient rats. *Physiol Behav* 72, 685-690.

Uneyama H, Niijima A, San Gabriel A & Torii K. (2006). Luminal amino acid sensing in the rat gastric mucosa. *Am J Physiol Gastrointest Liver Physiol* 291, G1163-1170.

Wang Y, Chandra R, Samsa LA, Gooch B, Fee BE, Cook JM, Vigna SR, Grant AO & Liddle RA. (2011). Amino acids stimulate cholecystokinin release through the Ca2+-sensing receptor. *Am J Physiol Gastrointest Liver Physiol* 300, G528-537.

Tables

Table 1

Ingredients and properties of the four L-lysine test meals for human application

Ingredients/properties	**units**	**values**			
LMH	[g]	0.5	1.2	3.0	7.5
NaCl	[g]	1.24	1.12	0.8	0.0
Evian® (still water)	[ml]	300			
Locus beam gum (E410)	[g]	3			
MR contrast agent (Dotarem®)	[µl]	350 µl(≙175 µmol ≙583.3 µM)			
Osmolarity	[mOsm/l]	152.52			
pH	pH	9.2	9.6	9.6	9.85
Caloric load	[kcal]	2.3	4.5	10.4	25.1

Figure captions

Figure 1

Schematic of the human study protocol. The schematic depicts the sequence of MRI measurements, venous blood sampling and visceral sensation ratings as performed in all three study sessions.

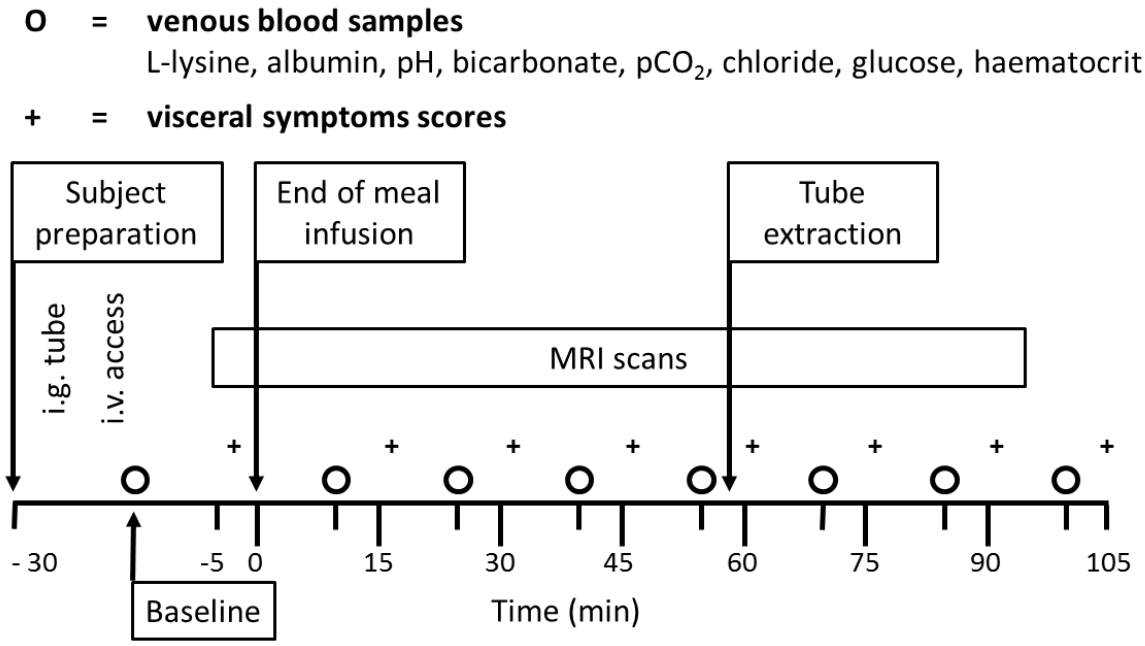


Figure 2

Dose dependent effect of intragastric L-lysine administration on GI wet weight in rats.

L-lysine dose-dependently increased stomach (A) and caecum wet weight (B) in rats. Individual dots represent wet weight measurements of individual animals treated with the respective L-lysine dose. The predicted linear dose-response curve is presented as black solid line.

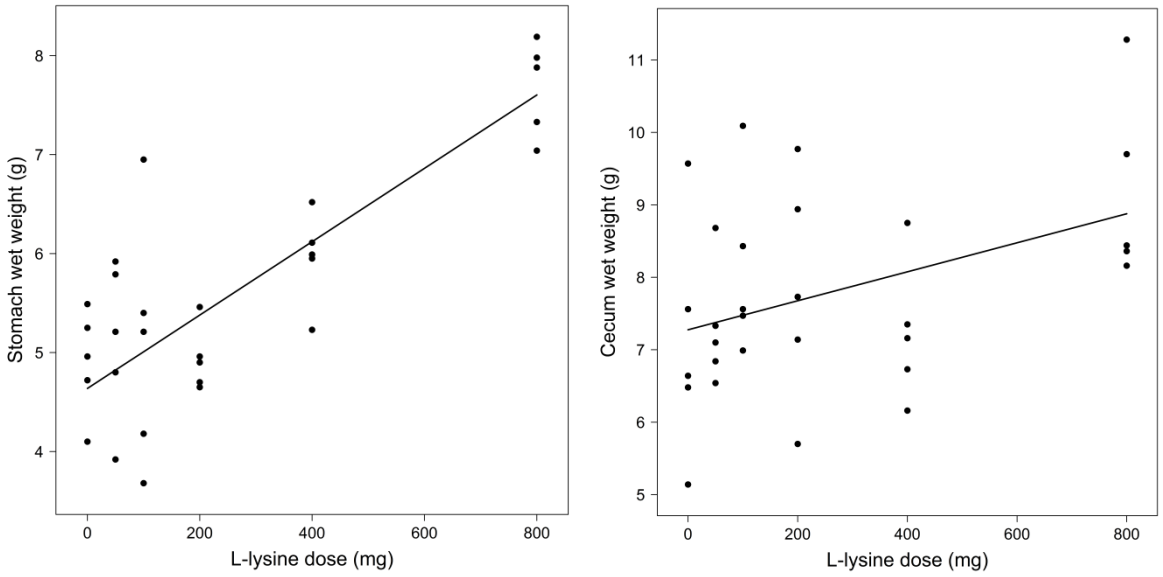


Figure 3

Dose dependent effect of intragastric L-lysine administration on phenol red content in rats. Phenol red content is expressed as percentage of the initially administered total phenol red amount. L-lysine dose-dependently increased gastric phenol red content (A) and decreased small intestinal (B) and colon (C) phenol red content in rats. Individual dots represent residual phenol red content measurements of individual animals treated with the respective L-lysine dose. The predicted linear dose-response curve is presented as black solid line.

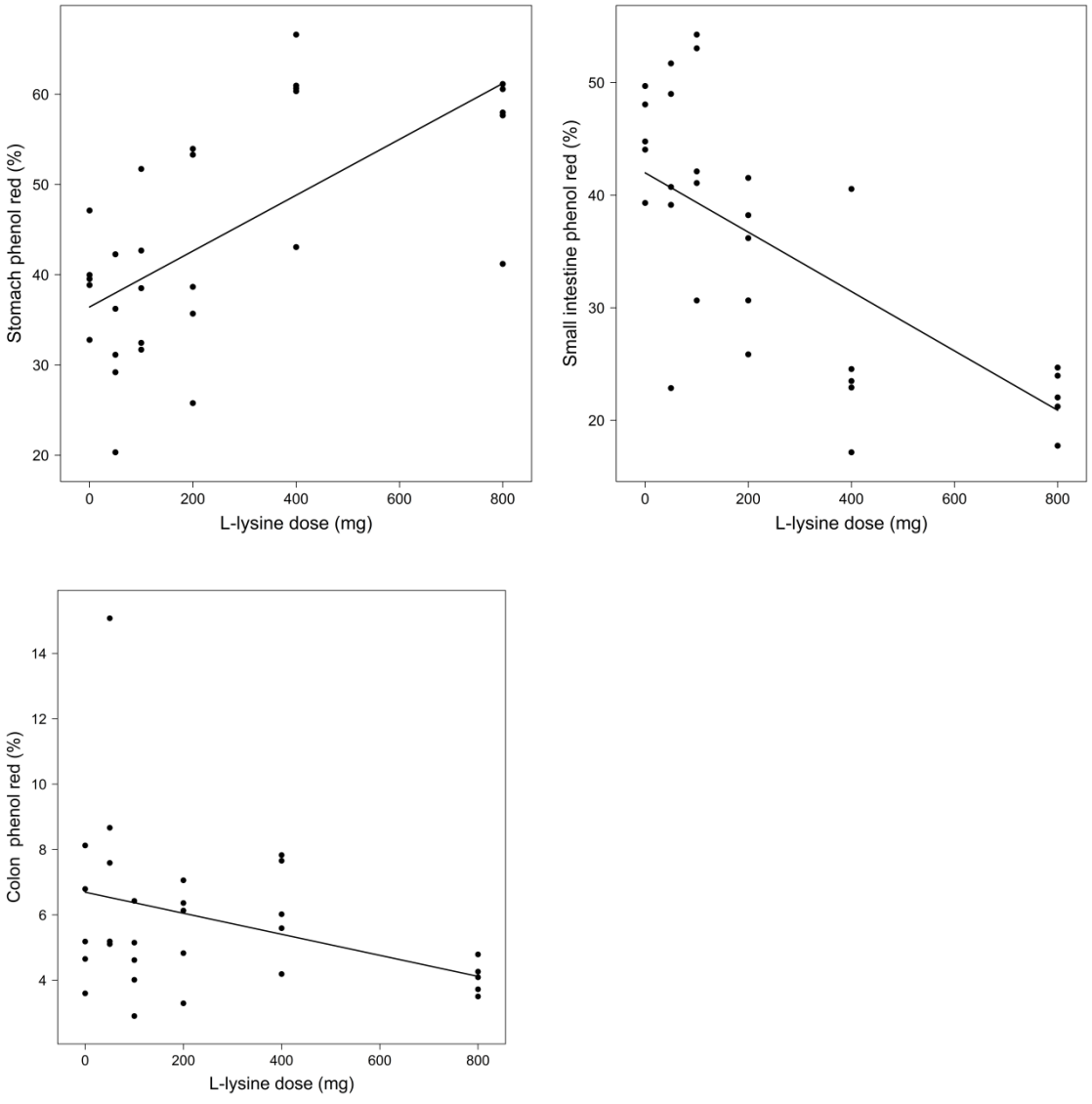


Figure 4

Dose dependent L-lysine concentrations in venous blood after intragastric application of L-lysine in humans. Data is expressed as delta over baseline (DOB) with baseline values measured before intragastric L-lysine infusion. (A) Smoothed DOB L-lysine concentration curves (lines) overlaid on the individual measurements (dots) of all human subjects each treated with three (of the total four) different L-lysine doses. L-lysine concentration data of one dose in subject PB1_1991 had to be dismissed due to subject non-compliance of study protocol. (B) The linearly predicted L-lysine concentration increase per dose (solid lines) overlaid on the individual measurements (dots).

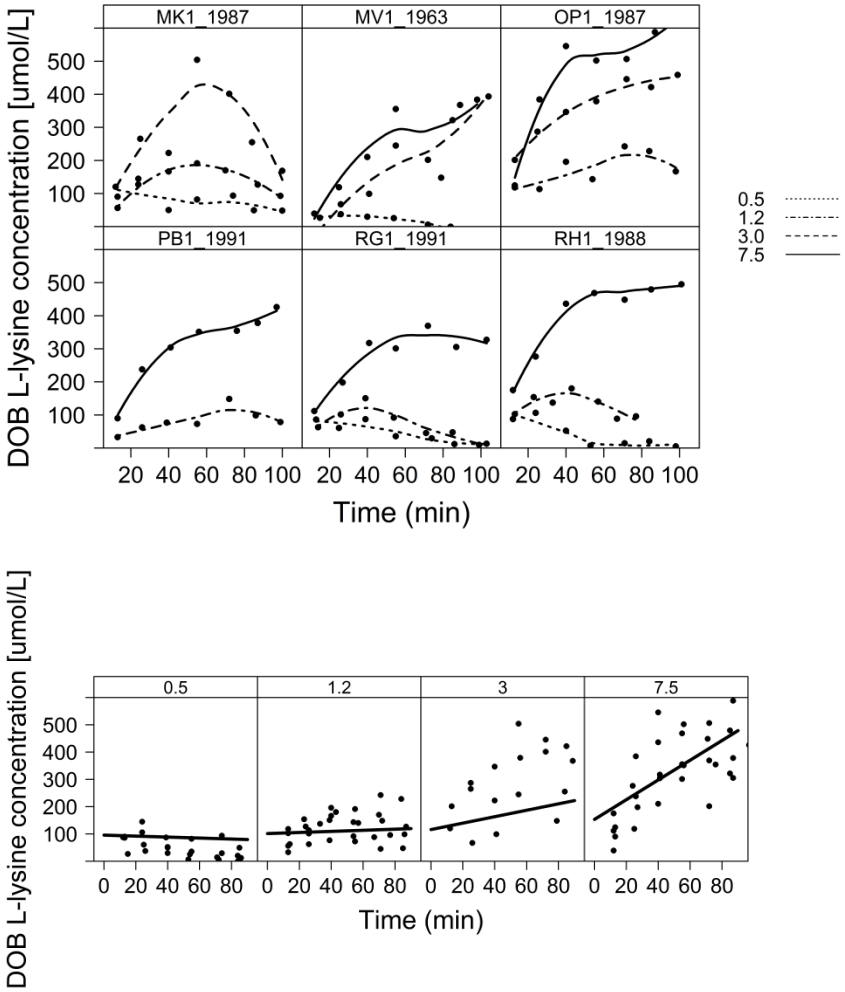
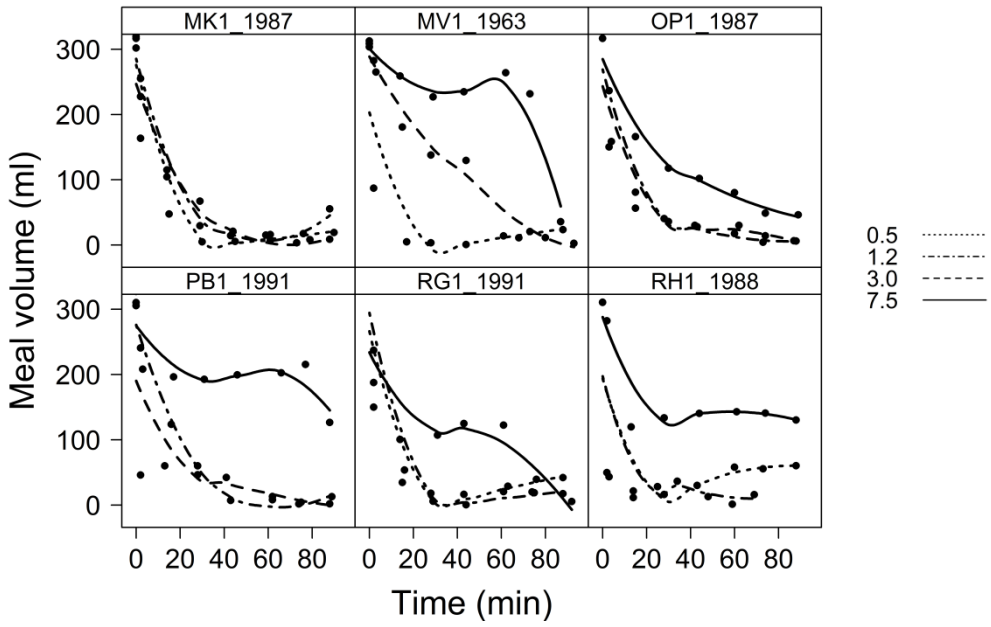


Figure 5

Dose dependent effect of intragastric L-lysine application on gastric emptying in humans. (A) Smoothed gastric emptying curves (lines) overlaid on the individual volume measurements (dots) of all human subjects each treated with three (of the total four) different L-lysine doses. (B) L-lysine dose-dependently increased t50 (computed from smoothed gastric emptying curves) in humans. Values of t50 (y-axis) are plotted in log10 scale, while L-lysine doses (x-axis) are plotted in log2.5 scale.



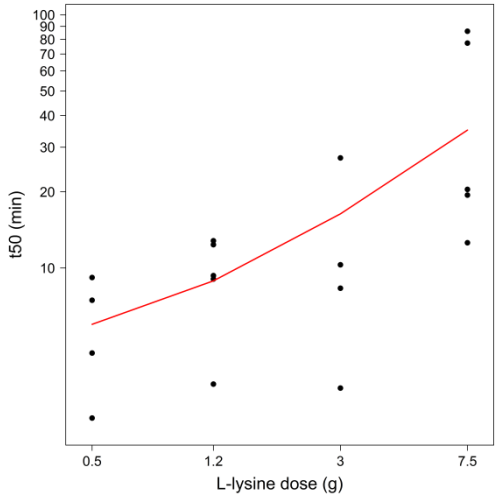


Figure 6

Dose dependent effect of intragastric L-lysine application on intestinal fluid volume in humans. (A) Smoothed intestinal fluid volume curves (lines) overlaid on the individual volume measurements (dots) of all human subjects each treated with three (of the total four) different L-lysine doses. (B) The linearly predicted intestinal fluid volume increase per dose (solid lines) overlaid on the individual measurements (dots).

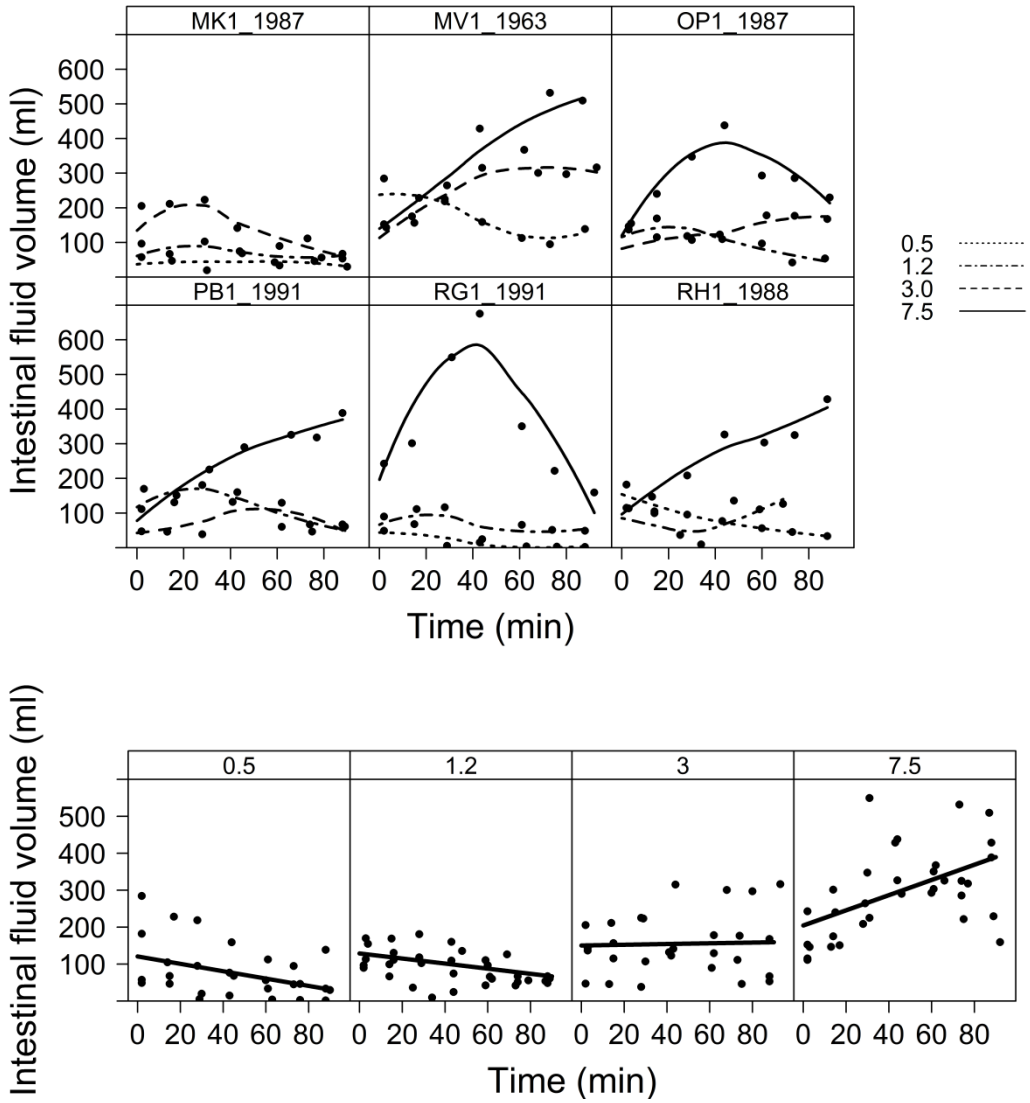
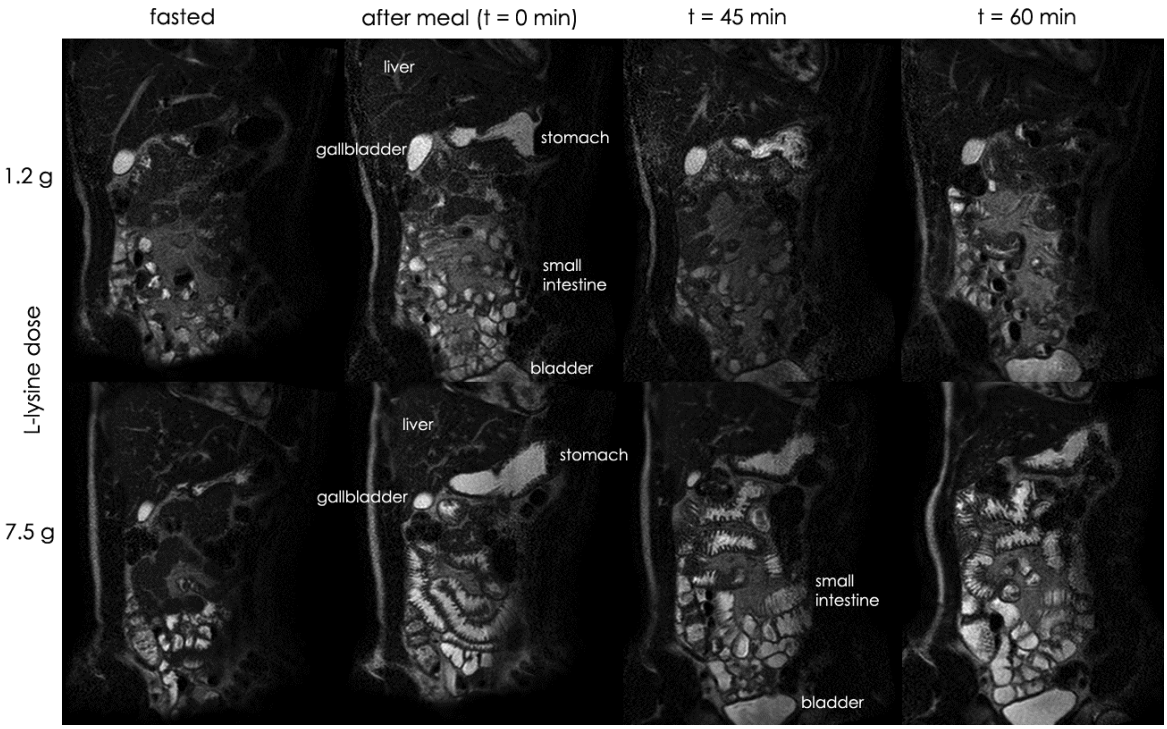


Figure 7



MR image data highlighting the dose dependent effect of intragastric L-lysine application on gastrointestinal fluid volume in humans. The difference in the accumulation of intestinal fluid volume and the delay in gastric emptying between L-lysine doses 1.2 g and 7.5 g is depicted over 60 min in representative MR image data of one subject (RH1_1988). Imaged GI organs and anatomical landmarks are indicated.

7. Unpublished Results

7.1 The Impact of L-arginine, L-lysine and L-glutamic acid on Circulating Gastrointestinal Hormone and Metabolite Concentrations

Rational

Important factors in the modulation of food intake and gastrointestinal function are gastrointestinal hormones as discussed in the introduction. They can act in a paracrine and/or endocrine manner. Locally they can directly alter gastrointestinal function and/or modulate vagal afferent firing. By circulation they can directly act on hormone sensitive central neurons and the vagus nerve among many others. The paracrine and the endocrine mechanism can regulate gastrointestinal motility and/or eating behavior. Hence, we hypothesized that L-arginine, L-lysine and L-glutamic acid differentially stimulate the release of gastrointestinal hormones and thereby indirectly inhibit food intake and modulate gastrointestinal motility. We assessed circulating gastrointestinal hormones in the portal vein and in general circulation. The portal vein is of particular interest because of its unique anatomical localization and the therefore expected high nutrient and hormone concentration as discussed in the introduction.

Methods

Animal housing, amino acid administration and dosing was performed as described in “Specific Amino Acids Inhibit Food Intake via the Area Postrema or Vagal Afferents”. To test the above hypotheses we conducted a terminal experiment focusing on one time point (30 min post application). Rats were food deprived for 12 h and received at dark onset an oral administration of a candidate amino acid. Glucose (isomolar) served as a positive control. Rats were returned to their home cage and had no access to food. 30 min post-administration rats were anesthetized and blood collected from the portal vein, the tongue and the heart by puncture with a syringe. Blood was collected into two EDTA-coated tubes. The tube for amino acid analysis was not supplemented, whereas the tube for hormone analysis was supplemented with 1 µl / 100 µl blood protease inhibitor (Sigma), 1 µl / 100 µl DPP IV (EMD Millipore) and 0.1 mg / 100 µl blood Perfabloc (Roche). We showed that supplementation interfered with amino acid analysis, but improves gastrointestinal hormone stability according to the kit manufacture. Blood was gently inverted, immediately centrifuged for 10 min at 1000 g and 4 °C. Plasma was collected and stored at -20 °C. Plasma amino acid concentrations were measured by the Functional Genomics Center Zurich using high pressure liquid chromatography, plasma glucose by AccuCheck Aviva (Roche) and plasma hormones using the Milliplex Map Kit – Rat Metabolic Magnetic Bead Panel based on the Luminex xMAP technology. Manufacture procedures were followed and magnetic sorting performed with a hand-held magnetic separation block. Importantly all samples were detected in the linear range of the standards and all quality control samples were in the manufacture target range. Amylin was below the detection range in all samples.

Results

All the data was listed and statistically analyzed comparing to water control (Table 1). Under all conditions the orally applied metabolites (glucose and individual amino acids) were absorbed, appeared in plasma of the portal vein and in general circulation (tongue/heart), and increased by several folds in concentrations indicating proper experimental application. The concentration in the portal vein was higher compared to common circulation for most applied metabolites. Different

metabolic products were as well detected dependent on the applied metabolite. For instance, L-arginine application increased the ornithine concentration. However, in our experimental conditions neither L-arginine, L-lysine nor L-glutamic acid triggered a measurable change in the concentration of a specific gastrointestinal hormone in the portal vein, heart or tongue plasma. Specifically, no significant change in the concentration of insulin, glucagon, glucose-dependent insulinotropic peptide (GIP), glucagon-like peptide-1 (GLP-1), amylin, ghrelin, leptin, pancreatic polypeptide (PP) and peptide tyrosine tyrosine (PYY) were detected after amino acid administration (Tab. 1). Glucose, the positive control, stimulated the release of GIP and a general reduction of circulating amino acids was observed compared to water control.

Conclusion

We conclude that the measured circulating hormones do not play a central role in mediating the anorectic and gastrointestinal responses reported for L-arginine, L-lysine and L-glutamic acid. This conclusion does not include a paracrine action of the hormones on vagal afferents or on the gastrointestinal tract. The power of this experiment is limited as it assessed only one time point and therefore neglects the temporal aspect of metabolite absorption and hormone release. Nevertheless the 30 min time point was a good choice, because after a meal most gastrointestinal hormones were elevated for at least 30 min and detectable with the here used method as shown by Shin et al. (138). Additionally our observed gastrointestinal hormone concentrations were in a comparable range to the basal concentrations measured by Shin et al. Our positive control, oral glucose application, stimulated the release of GIP but no change in insulin concentration was detected. This might have been expected as plasma insulin peaks 15 min after an oral glucose tolerance test, but returns to baseline quickly within 30-60 min (2). The here used glucose dose is “only” 25 % of the one used in a classical oral glucose tolerance test (4 g/kg), hence, differences might be explainable. Indeed insulin concentration drop to baseline after the application of 1 g/kg glucose in the rat. Nevertheless the increase in GIP concentration and the general decrease of amino acid concentration indicate a post-insulinic phase. Insulin is generally described to increase peripheral amino acids absorption in text books (157). Taken together, the data quality indicates a proper experimental approach but no amino acid induced release of circulating hormones.

This conclusion has to be taken with sufficient care, because negative data are difficult to interpret. Therefore next I briefly discuss specific limitations. First of all a defined set of hormones and metabolites was measured therefore an unknown compound or peptide may mediated the effect to the specific amino acids. Second, the measurement of hormones is a technical challenge. Normally, several splicing variants of one hormone exist and they might act by different receptors. They differ in stability, activity, half-life, etc. These differences were not accounted for by our measuring technique. Third, hormones are difficult compounds not only for measurement but as well for application. The problematic is best illustrated, if one considers that intraperitonal injection of even well-known anorectic hormones such as leptin, insulin, PYY or CCK do not always inhibit food intake. The mystery is why and was recently reviewed by two respected seniors in the field (168). Fourth, to correctly assess the above hypothesis feeding behavior and gastrointestinal function study should have been performed within hormonal inhibitor studies or even better within the specific gastrointestinal hormone knock-out mice if available. We did not follow this route because we did not find any indications for a candidate hormone to test in more detail. In sum, we did not detect amino acid induced changes in gastrointestinal hormones in the circulation. Therefore we propose

Table 1 – Plasma hormones and metabolites 30 min after individual amino acid administration.

Oral gavage of		H_2O			L-alanine			D-(+)-glucose			L-arginine			L-lysine			L-glutamic acid		
Measured Compound	**Location**	**Mean**	**SEM**	**p-value**	**Mean**	**SEM**	**p-value**	**Mean**	**SEM**	**p-value**	**Mean**	**SEM**	**p-value**	**Mean**	**SEM**	**p-value**	**Mean**	**SEM**	**p-value**
Hormones																			
GIP	tongue	25	9		28	4		67	10	**	24	4		13	5		18	2	
[pg/ml]	portal vein	67	14		56	15		127	23	***	39	6		36	4	*	57	9	
	heart	45	9		40	7		109	5	***	28	2		25	4		39	5	
Leptin	tongue	278	109		409	70		309	84		687	210	*	316	118		253	59	
[pg/ml]	portal vein	630	141		757	197		773	189		970	316		841	100		905	215	
	heart	714	161		807	175		1029	173		1121	224		1084	187		912	89	
Glucagon	tongue	1	0	na	nd	nd	na	1	0	na	2	1	na	5	4	na	1	0	na
[pg/ml]	portal vein	26	9		19	8		16	1		31	11		43	12		22	8	
	heart	12	0		2	0		3	1		13	9		13	7		3	0	
Insulin	tongue	942	540		963	282		468	74		1358	570		2157	731		1536	500	
[pg/ml]	portal vein	4594	1331		1922	811		2378	1527		4782	1664		5013	1163		6268	1431	
	heart	1394	486		1275	666		693	110		2365	1091		3377	1036		3044	743	
PP	tongue	nd	nd	na	nd	nd	na	nd	nd	na	nd	nd	na	nd	nd	na	nd	nd	na
[pg/ml]	portal vein	63	15		50	13		33	13		50	24		38	12		59	15	
	heart	nd	nd	na	nd	nd	na	nd	nd	na	nd	nd	na	nd	nd	na	30	0	na
PYY	tongue	nd	nd	na	nd	nd	na	nd	nd	na	nd	nd	na	nd	nd	na	nd	nd	na
[pg/ml]	portal vein	78	18	na	nd	nd		77	15	na	130	22	na	136	33	na	70	4	na
	heart	41	0	na	nd	nd		nd	nd	na	86	7	na	84	22	na	37	0	na
Grehlin	tongue	90	18		62	11		74	8		98	26		103	25		64	14	
[pg/ml]	portal vein	47	7		52	11		24	5		71	27		90	15		53	13	
	heart	55	16		43	8		32	15		80	30		67	18		51	14	
GLP-1	tongue	nd	nd	na	nd	nd	na	nd	nd	na	nd	nd	na	nd	nd	na	nd	nd	na
[pg/ml]	portal vein	177	58		87	20		73	35		264	61		301	96		196	47	
	heart	63	10	na	nd	nd	na	21	0	na	nd	nd	na	nd	nd	na	nd	nd	na
Amino Acids																			
Alanine	tongue	602	28		3433	115	***	571	36		703	62		658	24		862	91	
[µmol/l]	portal vein	833	80		3374	113	***	829	60		1093	104		918	106		1311	86	***
	heart	678	43		3546	212	***	665	66		774	37		647	41		953	73	
Arginine	tongue	123	12		120	8		77	18		922	62	***	140	10		110	12	
[µmol/l]	portal vein	69	19		47	14		39	12		1000	178	***	118	18		89	9	
	heart	122	14		124	15		94	11		787	115	***	134	11		113	9	
Asparagine	tongue	107	8		133	6		80	4		96	7		115	5		93	8	
[µmol/l]	portal vein	116	12		143	10		107	9		116	11		158	20	**	107	10	
	heart	116	10		146	8		93	7		97	6		110	3		103	5	
Aspartic Acid	tongue	12	1		25	3		16	3		15	2		15	1		66	13	**
[µmol/l]	portal vein	41	7		99	10	**	42	4		57	14		32	10		130	29	***
	heart	29	4		54	11		28	8		36	7		22	3		98	26	***
Cysteine	tongue	7	2		8	2		4	1		10	2		12	2		8	2	
[µmol/l]	portal vein	11	3		8	2		6	2		15	2		15	3		13	2	
	heart	11	3		10	2		7	1		12	0		14	2		12	2	
Glutamine	tongue	1101	58		1223	54		886	48		1040	89		993	46		1144	104	
[µmol/l]	portal vein	1158	114		1084	80		1039	58		1041	121		923	94		1373	128	
	heart	1428	110		1478	80		1148	89		1167	56		1204	37		1464	67	
Glutamic Acid	tongue	133	10		211	17		148	28		186	18		163	11		775	174	**
[µmol/l]	portal vein	585	123		1140	97	*	695	131		781	217		484	200		1527	341	***
	heart	204	30		358	57		227	57		340	52		277	39		964	260	***
Glycine	tongue	512	36		538	28		315	62	**	447	45		555	40		433	27	
[µmol/l]	portal vein	472	91		466	36		445	18		525	50		662	77	*	513	28	
	heart	566	42		577	38		436	14		479	32		593	40		518	21	
Histidine	tongue	65	7		74	5		51	4		53	3		56	3		57	4	
[µmol/l]	portal vein	77	10		80	7		65	4		67	4		70	2		70	6	
	heart	78	10		86	7		59	5		58	5		63	2		69	4	
Isoleucine	tongue	83	4		64	2		57	8		75	4		84	5		90	25	
[µmol/l]	portal vein	160	20		120	4		137	15		151	22		157	13		132	23	
	heart	106	10		86	4		80	12		104	9		101	6		92	11	
Leucine	tongue	155	7		116	5		106	15		155	6		159	9		163	43	
[µmol/l]	portal vein	287	36		219	9		251	26		301	38		302	26		241	42	
	heart	198	19		160	7		150	22		212	15		197	12		171	18	
Lysine	tongue	327	46		372	44		261	27		372	39		2804	146	***	368	57	
[µmol/l]	portal vein	403	39		432	41		316	32		407	52		3953	358	***	423	61	
	heart	354	48		434	50		306	44		403	57		2835	191	***	389	43	
Methionine	tongue	45	2		40	2		36	2		47	2		55	3		41	4	
[µmol/l]	portal vein	60	7		50	2		51	4		60	4		73	3	*	52	4	
	heart	54	4		48	2		42	4		51	4		57	2		46	2	
Phenylalanine	tongue	50	5		42	3		41	6		48	1		48	2		51	9	
[µmol/l]	portal vein	94	19		78	8		85	8		89	12		88	8		77	14	
	heart	65	9		56	4		51	6		61	9		62	4		60	7	
Proline	tongue	161	11		195	11		128	6		218	14	**	172	14		163	11	
[µmol/l]	portal vein	169	15		187	13		142	6		276	18	***	217	22	*	197	14	
	heart	175	17		206	12		139	9		217	14		172	9		178	10	
Serine	tongue	286	26		382	23		173	34	*	255	18		270	13		259	18	
[µmol/l]	portal vein	276	66		354	31		242	14		267	25		303	27		307	25	
	heart	313	42		409	30		233	9		249	24		276	12		297	16	
Threonine	tongue	242	16		276	15		173	14		217	17		256	29		223	23	
[µmol/l]	portal vein	274	29		286	21		206	8		244	24		308	34		268	24	
	heart	269	27		303	19		191	8	*	218	20		266	21		251	17	
Tryptophan	tongue	60	10		56	5		47	3		57	8		67	4		53	4	
[µmol/l]	portal vein	67	12		64	5		54	3		66	7		75	5		64	7	
	heart	63	12		61	5		50	3		56	7		68	4		61	8	
Tyrosine	tongue	53	7		45	4		36	4		49	3		57	3		53	9	
[µmol/l]	portal vein	90	19		72	7		68	7		84	10		86	7		78	14	
	heart	66	11		57	5		44	4		61	4		65	3		61	9	
Valine	tongue	187	6		158	6		134	10		175	9		185	11		192	41	
[µmol/l]	portal vein	307	36		237	7		247	22		291	32		292	20		259	34	
	heart	225	17		193	6		168	19		221	12		217	10		197	13	

Oral gavage of		H_2O			L-alanine			D-(+)-glucose			L-arginine			L-lysine			L-glutamic acid		
Measured Compound	**Location**	**Mean**	**SEM**	**p-value**	**Mean**	**SEM**	**p-value**	**Mean**	**SEM**	**p-value**	**Mean**	**SEM**	**p-value**	**Mean**	**SEM**	**p-value**	**Mean**	**SEM**	**p-value**
Non-Amino Acids																			
Glucose	tongue	156	13		159	14		103	9		179	11		197	7		286	66	
[mg/dl]	portal vein	171	44		441	45	***	395	75	**	244	16		318	81		299	72	
	heart	203	39		230	29		205	42		281	22		258	27		243	28	
1-Methylhistidine	tongue	1.0	0.2		1.5	0.1		0.8	0.1		1.4	0.1		1.7	0.2		2.2	0.5	*
[µmol/l]	portal vein	1.9	0.3		1.7	0.2		1.2	0.2		1.2	0.3		2.0	0.3		2.2	0.8	
	heart	1.1	0.2		1.8	0.2		1.0	0.1		1.3	0.3		2.0	0.3		1.5	0.3	
3-Methylhistidine	tongue	3.9	0.5		5.0	0.3		3.5	0.3		3.3	0.1		0.8	0.3		3.5	0.6	
[µmol/l]	portal vein	3.3	0.6		5.0	0.4		3.3	0.4		8.6	4.2	**	1.1	0.5		4.1	0.6	
	heart	4.2	0.4		5.2	0.4		3.7	0.4		4.2	0.7		1.5	0.5		4.5	0.6	
Alpha-Amino-N-Butyric Acid	tongue	14	2		14	1		9	1		12	2		12	3		12	3	
[µmol/l]	portal vein	24	5		16	2		11	1	***	17	3		20	3		15	3	*
	heart	15	3		16	2		10	1		16	3		16	3		13	2	
Alpha-Aminodipic Acid	tongue	1.0	0.4		2.2	0.1		0.9	0.4		2.0	0.6		14.7	1.9	***	3.6	0.8	*
[µmol/l]	portal vein	3.2	0.4		4.2	0.1		3.3	0.4		3.8	0.7		16.7	1.1	***	6.2	1.1	*
	heart	2.1	0.1		2.8	0.3		1.3	0.6		3.2	0.6		16.1	1.4	***	5.2	0.8	*
Beta-Alanine	tongue	10	1		11	2		11	2		12	2		14	1		11	1	
[µmol/l]	portal vein	10	2		11	2		10	1		13	3		12	2		13	2	
	heart	11	2		12	2		12	2		13	2		14	1		13	1	
Carnosine	tongue	4.1	1.6		4.6	1.7		3.5	1.0		8.6	1.4		2.4	0.3		3.2	0.6	
[µmol/l]	portal vein	5.2	1.6		4.9	0.8		9.4	2.0		24.3	6.4	***	7.2	2.6		8.1	1.8	
	heart	4.2	0.7		2.7	0.3		3.7	0.7		10.9	1.0		4.9	1.6		4.1	0.7	
Citrulline	tongue	115	9		145	11		91	7		115	11		107	10		108	10	
[µmol/l]	portal vein	141	14		182	13	**	115	3		154	11		135	7		137	11	
	heart	124	13		157	11		96	2		117	8		113	8		111	7	
Cystathionine	tongue	1.7	0.2		2.0	0.3		1.7	0.1		1.6	0.3		1.8	0.1		2.4	0.3	
[µmol/l]	portal vein	2.0	0.2		2.3	0.3		1.9	0.1		2.0	0.2		2.0	0.4		2.9	0.6	
	heart	2.0	0.2		2.6	0.4		1.9	0.1		1.9	0.3		1.8	0.3		3.1	0.4	*
Ethanolamine	tongue	16	1		18	1		17	2		14	2		16	1		22	5	
[µmol/l]	portal vein	33	6		40	4		38	3		31	8		27	4		36	7	
	heart	24	2		28	3		24	3		25	3		22	2		27	3	
GABA	tongue	1.8	0.4		2.4	0.3		1.6	0.3		2.0	0.5		1.6	0.5		2.0	0.4	
[µmol/l]	portal vein	2.0	0.4		3.4	0.5		2.2	0.5		3.0	0.6		2.4	0.1		3.0	0.3	
	heart	1.8	0.5		2.8	0.3		1.9	0.4		2.7	0.2		2.1	0.5		2.4	0.5	
Hydroxyproline	tongue	62	7		84	6		52	3		60	3		71	10		59	8	
[µmol/l]	portal vein	43	10		70	7	*	47	3		53	5		59	10		66	6	
	heart	69	8		91	8		56	4		57	2		68	7		57	12	
Ornithine	tongue	75	6		90	4		84	15		567	62	***	59	10		121	25	
[µmol/l]	portal vein	169	16		212	19		170	26		1081	126	***	84	17		186	24	
	heart	97	9		109	12		83	15		688	32	***	85	14		129	12	
Phospohoethanolamine	tongue	15	2		19	5		17	2		15	1		18	1		23	3	
[µmol/l]	portal vein	25	3		35	4		29	6		31	6		23	4		36	6	
	heart	16	1		18	3		16	2		17	1		17	1		25	3	
Sarcosine	tongue	15	1		13	2		9	3		13	2		13	2		15	2	
[µmol/l]	portal vein	20	3		16	2		14	1		21	2		20	5		17	2	
	heart	11	1		12	2		13	1		13	3		15	2		17	1	
Taurine	tongue	187	17		230	27		208	24		212	19		222	22		232	49	
[µmol/l]	portal vein	233	40		276	46		288	56		284	19		232	24		216	25	
	heart	434	75		300	22		379	90		281	24		269	31	*	339	47	
Unknown Compound	tongue	49800	5170		60522	6069		41531	4326		52656	5149		401341	52757	***	56616	8642	
[area under the peak]	portal vein	74355	9288		85102	9147		49259	11141		64734	6702		570966	96183	***	89575	9108	
	heart	55138	5162		68650	8624		42458	4237		64253	10153		356618	28216	***	61986	3152	

n= 6; statistical comparison made to H_2O; unpaired two-way ANOVA, Bonferroni post test

nd=not detected na=not accessed

that L-arginine, L-lysine and L-glutamic acid inhibit food intake and modulate gastrointestinal motility independent of circulating hormones. This conclusion is as well supported by the behavioral data shown in the research article"Specific Amino Acids Inhibit Food Intake via the Area Postrema or Vagal Afferents.". All three amino acids induced satiation by reducing first meal size, but not meal duration. This is in contrast to classical satiation-inducing peptides such as CCK or amylin which proportionally reduce meal size and duration (167). Additionally, the anorectic effect of CCK for instance is compensated by hyperphagia as soon as the hormone loses its activity (degradation, stability,…). In contrast, L-arginine and L-lysine decreased food intake as well in the long term, whereas L-glutamic acid treated animals compensated for the initial decrease in food intake in the following 48 h. Lastly several gastrointestinal hormones mediate their anorectic effect by vagal afferents (see introduction). L-arginine and L-glutamic acid still induced an anorectic behavior in capsaicin de-afferented rats. Hence, several lines of experiments indicate no involvement of circulating gastrointestinal hormones for the anorectic effect induced by L-arginine, L-lysine and L-glutamic acid. This conclusion is only valid for the anorectic effect and not for the amino acid specific impact on gastrointestinal function.

7.2 The Impact of L-arginine, L-lysine and L-glutamic acid on Defecation

Rational

The data presented in the manuscript "Specific Amino Acids Inhibit Food Intake via the Area Postrema or Vagal Afferents." could as well be interpreted in the following manner as proposed in the 1.5 year committee meeting. The amino acid L-arginine and L-lysine stimulated a putative alarm system controlling amino acid homeostasis, which protects the *milieu interieur* from an amino acid overload. The applied doses of L-arginine and L-lysine were relatively high (Table 2 – see discussion, 113 %, 104 % of their specific daily intake), which led to a strong raise in their plasma concentration (Table 1; fold changes relative to H_2O– L-alanine [4-6x],L-arginine [6-15x], L-lysine [8-10x], L-glutamic acid [2.5-6x]) and these could be detected by a alarm mechanism. The immediate protective mechanism induced will target to reduce nutrient absorption in the small intestine. Luminal nutrients would be removed by increased intestinal motility, their concentration diluted by increased secretion and their delivery to the small intestine delayed by reduced gastric emptying. Additionally, food intake would be restricted and the animal will learn to avoid this nutrient sources reflected in the positive conditioned taste aversion test after oral application. Indeed L-arginine and L-lysine did trigger the above response. Additionally in humans' high doses of L-lysine induced diarrhea. L-alanine and L-glutamic acid (Table 2, 66 %, 27 % of daily consumption) did not elicit the alarm system because they are key energetic shuttling molecules between organs and had no malaise effect in oral conditioned taste aversion test. If there is such a putative protective mechanism aiming to avoid nutrient absorption by altering gastrointestinal functions, one would expect altered defecation specifically triggered by L-arginine and L-lysine as a mean to release over-counting nutrients. To test this hypothesis, we assessed defecation after individual amino administration.

Methods

Animal housing, amino acid administration and dosing was performed as described in "Specific Amino Acids Inhibit Food Intake via the Area Postrema or Vagal Afferents.". Animals were adapted to a feeding schedule enabling food intake only in the first 8 h after dark onset. Water was available ad libitum. After 16 h food deprivation rats were gavaged with an isomolar dose of individual amino acids including phenol red (1.5 mg) and 30 min later received access to food. Food intake was measured every 2 h. Feces were individually collected and counted every 2 h, weighted, and dried at 80 °C. Phenol red (Sigma) was extracted and quantified as described by others (153).

Results

L-arginine, L-lysine and L-glutamic acid reduced food intake in the first 2 h after amino acid application (Fig. 3A). No significant differences between treatment groups were found on feces number, wet weight or water content (Fig. 3B, C, E). A higher dry feces weight between 6-8 h was measured after L-lysine administration, but no other time point or treatment showed a significant effect (Fig. 3D). The administered phenol red, a spectroscopically detectable dye, was mainly recovered within feces samples after 4-8 h of administration and no treatment differences were found (Fig. 3F).

Conclusion

L-arginine, L-lysine, and L-glutamic acid inhibited short-term food intake as previously described in our manuscript entitled "Specific Amino Acids Inhibit Food Intake via the Area Postrema or Vagal Afferents.". No significant impact of L-arginine, L-lysine, and L-glutamic acid was found on defecation

or on the water content in the rat feces, which could indicate a very mild form of diarrhea. Interestingly the orally administered dye, phenol red, is recovered in the feces sample mainly after 4 h showing no faster gastrointestinal dye transit between treatment groups. This is in agreement with previous reports showing that an orally applied randomly labeled ^{14}C protein and a non-absorbable marker $^{51}CrCl_3$ reached the colon within 4 h (36). This collectively indicates that L-arginine, L-lysine and L-glutamic acid did not induce accelerated defecation nor changed total gastrointestinal dye transit time. The alternative interpretation of the gastrointestinal phenotype induced by L-arginine, L-lysine and L-glutamic acid is that they serve as direct sensory input to assess dietary protein content and quality *in vivo* enabling to adapt food processing and intake accordingly. This interpretation is part of the manuscript entitled "Specific Amino Acids Inhibit Food Intake via the Area Postrema or Vagal Afferents.".

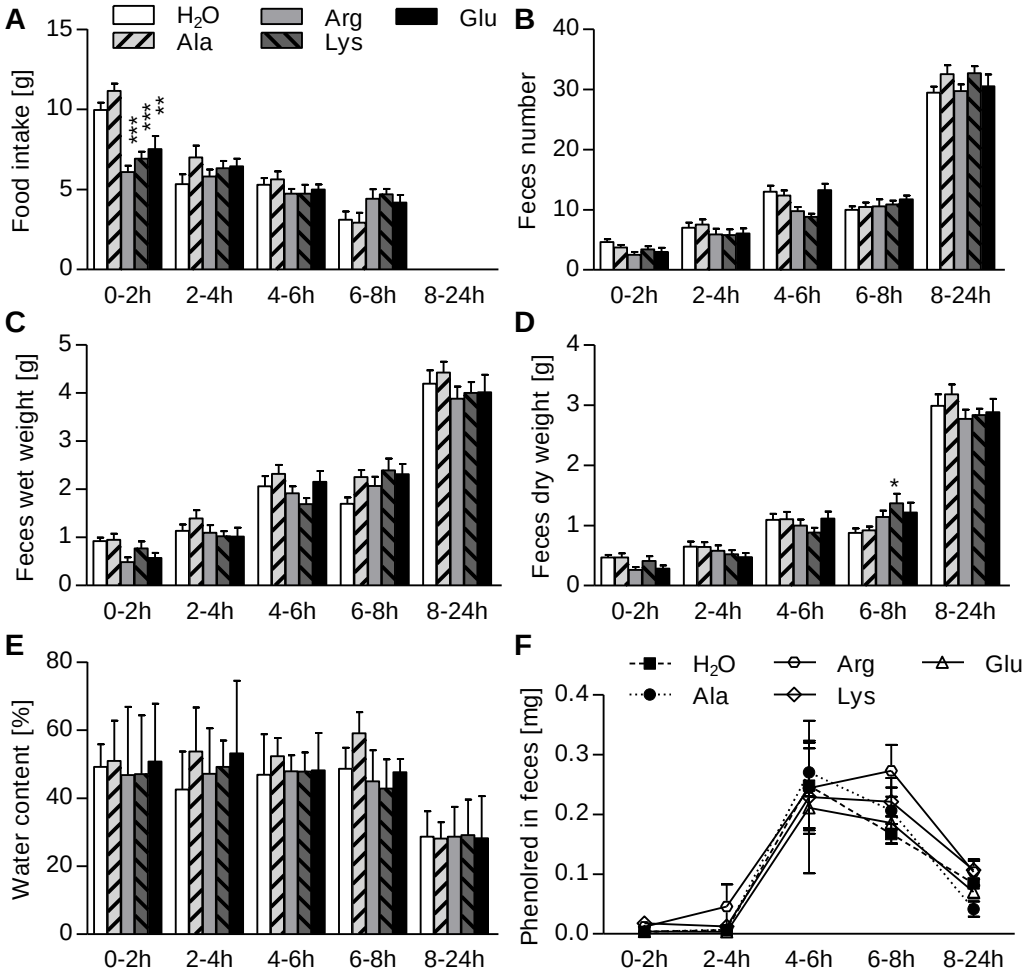


Figure 3 – Food intake and defecation following amino acid administration. After 16 h food deprivation animals were gavaged with an isomolar dose of individual amino acids including phenol red and 30 min later received access to food. Every following 2 h food intake was measured (A), feces collected and counted (B), weighted (C), dried (D) and water content calculated (E). Phenol red content of feces was measured (F). Values are mean ± sem; n = 10-12, unpaired two-way ANOVA, Bonferroni Post-Test, **P<0.05, **P<0.01, ***P<0.001.

7.3 The Impact of L-arginine, L-lysine and L-glutamic acid on Body Weight in the Short Term

Rational

As L-arginine, L-lysine and L-glutamic acid are potent inducer of satiation, we hypothesized they could be applied to reduce or limit body weight gain. As discussed in the introduction, high protein diets such as the Atkins diet are of broad interest, because they seem to selectively reduce fat mass and not alter lean body mass or energy expenditure (3, 75). Dietary supplementation with L-arginine revealed promising results in Zucker rats reducing body weight by 6 %, 10 %, and 16 % after 4, 7, and 10 weeks after treatment initiation (51, 98). The dietary supplementation with monosodium-glutamate or L-lysine was not successful in reducing body weight in rats (18, 152). Obviously these conclusions are restricted to the applied doses and to a constant increase of the supplemented amino acid. In this work, we always assessed the acute impact of a single amino acid dose on food intake. Hence there is a peak of amino acid concentration in plasma and not a constant supplementation potentially marking a conceptual difference. Therefore, we hypothesized that L-arginine, L-lysine and L-glutamic acid can limit body weight gain acutely.

Methods

Animal housing, amino acid administration and dosing was performed as described in “Specific Amino Acids Inhibit Food Intake via the Area Postrema or Vagal Afferents.”. Animals were adapted to a feeding schedule enabling food intake in the first 8 h after dark onset. Water was available ad libitum. At dark-onset animals were weighted and gavaged with an isomolar dose of individual amino acids. 30 min post application animals received access to food. Food intake was measured after 1 h to assess the short-term impact and after 8 h to assess the daily intake. During the first 3 days of the experiment all animals received a water gavage followed by 5 days of treatment with the respective amino acid.

Results

All animals allocated to the different treatment groups increased body weight by around 4.5 g/day during the initial treatment with water (Fig. 4A). Short-term and daily food intake did neither differ between treatment groups during the initial water treatment. Oral L-arginine and L-lysine reduced short term food intake in the first hour after application on every treatment day expect on day 4 (Fig. 4B). Daily food intake was not significantly reduced between treatment groups nor did we observe a significant impact on body weight gain (Fig. 4A, 4C).

Conclusion

L-arginine and L-lysine inhibited short-term food intake as previously described in our manuscript entitled “Specific Amino Acids Inhibit Food Intake via the Area Postrema or Vagal Afferents.” on day 1 and the following days expect for day 4. Surprisingly L-glutamic acid did not reduce food intake significantly. This divergence from previous findings might emerge, because the water and L-alanine group did eat less compared to previous experiments (normally +- 8 g in the first hour; +- 30 g/day). An intuitive explanation might be the stress induced by daily gavaging, however we did not find that oral applications impaired normal rat feeding or drinking behavior in our experience. Nevertheless, no significant impact of L-arginine, L-lysine, and L-glutamic acid was found on total food intake or on body weight gain thereby limiting the potential for an anti-obesity treatment. Even though this intervention for 5 days was not successful a more long-term application might be an alternative

strategy. The option would range from supplementing the water bottles with the candidate amino acids to the use of mini-pumps for a constant increase in plasma L-arginine, L-lysine and L-glutamic acid concentration. We did not continue on this avenue because the data was for us not promising enough. We concluded that the effect of the three individual amino acids most probably is limited to the short-term.

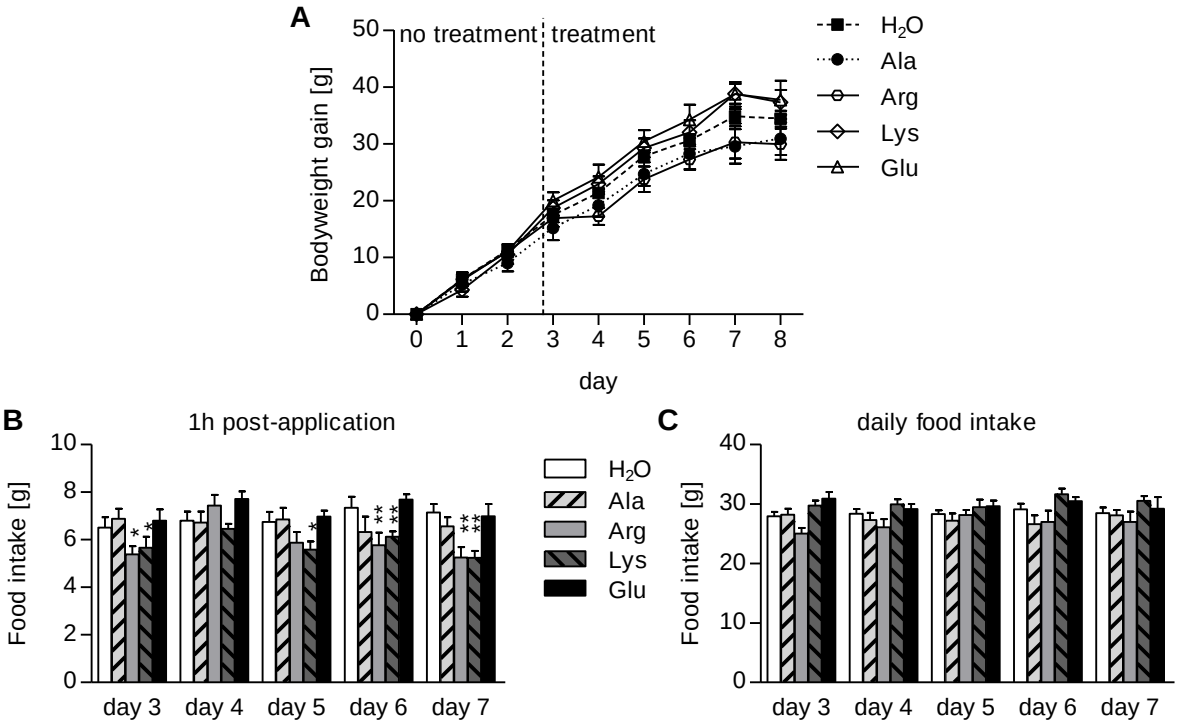


Figure 4 – Body weight gain and food intake of animals treated every day with a single individual amino acid. *(1) Animals were orally gavaged with H_2O from day 0 to day 2 and with the indicated amino acid from day 3 to day 7. (B) Food intake measured 1 h after amino acid application. (C) Food intake measured 8 h after amino acid application. Values are mean ± sem; n = 10, unpaired two-way ANOVA, Bonferroni Post-Test, *P<0.05, **P<0.01.*

7.4 The Impact of All Different Combinations of L-arginine, L-lysine and L-glutamic acid on the Control of Food Intake

Rational

From the experiment assessing the impact of L-arginine, L-lysine and L-glutamic acid on body weight we concluded that the effect of the three individual amino acids most probably is not strong enough to reduce food intake to the extent required for an impact on body weight. This raised the question, what high protein diets contain to mediate their long-term effect. We proposed that the combination of all individual amino acids might generate a combined effect. We therefore tested, if combinations of L-arginine, L-lysine and L-glutamic acid effect induce a stronger anorectic effect than an individual.

Methods

Animal housing, amino acid administration and dosing was performed as described in "Specific Amino Acids Inhibit Food Intake via the Area Postrema or Vagal Afferents.". Animals were adapted to a feeding schedule enabling food intake only in the first 8 h after dark onset. Water was available ad libitum. At dark-onset animals were weighted and gavaged with a combination of individual amino acids. 30 min post application animals received access to food. Food intake was measured as indicated after 1 h, 24 h and 48 h.

Results

To test this hypothesis we administered all three candidate amino acids in all possible combinations. The combination of L-arginine + L-lysine reduced short-term and long-term food intake and 24 h body weight gain most potently compared to all other combinations tested (Fig. 5A, 5B). Counter intuitively the addition of L-glutamic acid buffered or inhibited the anorectic effect of L-arginine, L-lysine and their combination. To further characterize the combined effect of L-arginine + L-lysine we conducted a dose response study (Fig. 5C). The combination did induce an anorectic effect at lower doses compared to the single. At higher doses no significant difference between single and the combination were measured. As all experiments so far ignored the osmolarity differences, we conducted a study under isomolar conditions. Isomolarity was achieved by adding L-alanine, an amino acids with no apparent effect on food intake. Only the combination L-arginine and l-lysine reduced food intake at the low dose of 5.4 mmol/kg, whereas the combination seemed to induce a similar effect as the single individual amino acid at the high dose of 13.6 mmol/kg (Fig. 5D, 5E).

Conclusion

We hypothesized that L-arginine, L-lysine and L-glutamic acid may combine to generate a stronger anorectic effect. Indeed the combination of L-arginine and L-lysine seemed to be more efficient in reducing body weight compared to other combinations. Further efforts aimed to characterize this combination and experimentally demonstrate synergism (53). Unfortunately these efforts were not successful at high doses (Fig. 5E). However, most striking and confusing was the finding that L-glutamic acid inhibits or even buffered the anorectic effect of L-arginine, L-lysine or their combination. We currently do not have a good explanation for this finding.

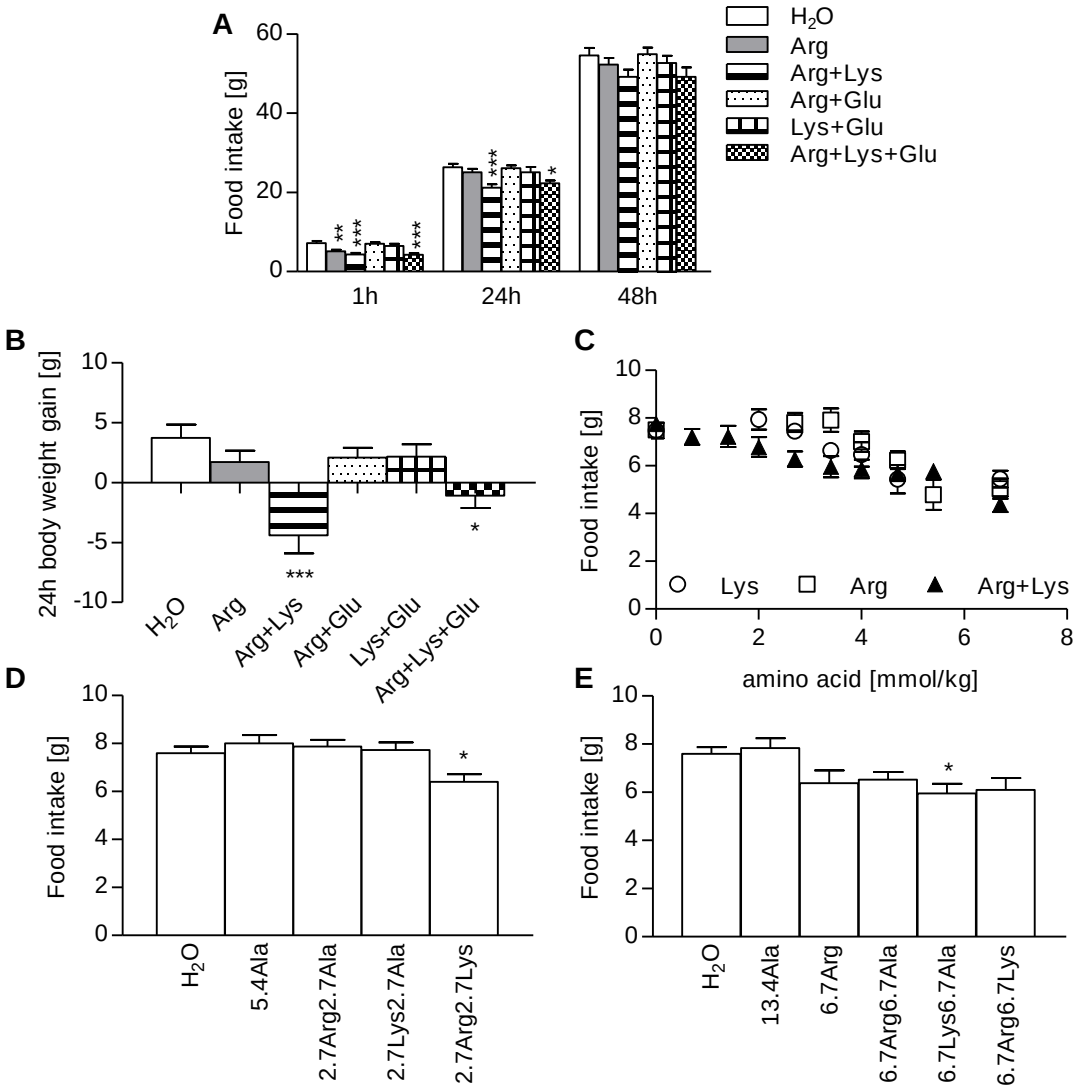


Figure 5 – The combined effect of L-arginine, L-lysine and L-glutamic acid on food intake. (A-B) Animals were orally gavaged with a combination of individual amino acids each at a fix dose of 6.7 mmol/kg. Food intake and body weight was measured. Values are mean ± sem; n = 10, unpaired one-way ANOVA, Dunnett Post-Test, *P<0.05, **P<0.01, ***P<0.001. (C) Animals were orally gavaged with different doses of individual or combination of amino acids and their subsequent food intake measured for 1 h. Values are mean ± sem; n = 10. (D-E) Animals were orally gavaged with a combination of individual amino acids at a isomolar dose of 5.4 mmol/kg or 13.4 mmol/kg and their subsequent food intake measured for 1 h. Values are mean ± sem; n = 11, unpaired one-way ANOVA, Dunnett Post-Test, *P<0.05.

7.5 Systematic Study of the Impact of Individual Amino Acids on Gastric Secretion and Emptying – a Novel Computed Tomography Study

Rational

Upon meal ingestion, gastrointestinal function can quickly adapt to the meal. This dynamic plasticity is based on mechanisms responding to meal born signals. They are to some extent autonomous but also controlled by the central nervous system via intertwined neuronal and endocrine signals. The afferent input can be mechanic or chemospecific. Most prominently gastric emptying was suggested to be calibrated to a fix emptying rate of 2-2.5 kcal/min in humans and of 0.03-0.045 kcal/min in rats (70, 96). However, others suggested an even more chemo-specific control of gastrointestinal function. For instance, a high protein diet delayed gastric emptying to a larger extends than the isocaloric control in humans and rats (13, 44). So far no study systematically addressed the effect of all 20 proteogenic amino acids on gastrointestinal function, but our recent efforts demonstrated a distinct impact of L-arginine, L-lysine and L-glutamic acid on gastric emptying, secretion and intestinal motility in rats. Here, we tested the impact of the other 17 proteogenic amino acids.

Methods

Animal housing, image acquisition and data analysis was performed as described in “Simultaneous assessment of gastric emptying and secretion in rats by a novel computed tomography based method”. Intragastrically administered L-amino acids and D-(+)-glucose (freshly prepared, isomolar, 6.7 mmol/kg) were applied in 2 ml tap-water containing 400 mg Sodium-diatrizoate. After 4 h food deprivation rats were gavaged with their respective dose and after application rats were returned to their home-cage. They did not have access to water or food after application. In the first experiment animals were imaged after 20 min and in the second experiment after 30 min using the computed tomography method described in the manuscript.

Results

H_2O is known to empty exponentially and significantly faster compared to caloric liquids. Therefore in this experiment D-(+)-glucose served as a negative control. The statistical comparison was made to L-alanine, because it is an amino acid which showed no impact on gastric secretion and emptying compared to glucose. Gastric emptying is delayed only by L-cysteine 20 min after administration, whereas L-methionine, L-arginine, L-tryptophan, L-lysine and L-cysteine showed increased stomach contrast agent content after 30 min (Fig. 6A, 6D). Total stomach volume and non-administered stomach volume increased after L-arginine and L-lysine administration and decreased after H_2O application after 20 min indicating altered gastric secretion (Fig. 6B & C). Total stomach volume was increased by L-methionine, L-arginine, L-tryptophan, L-lysine and L-cysteine and decreased for L-tyrosine and H_2O 30 min after their application (Fig. 6E). The non-administered stomach volume was increased only by L-arginine and L-lysine and decreased after L-tyrosine and H_2O administration 30 min post administration (Fig. 6F).

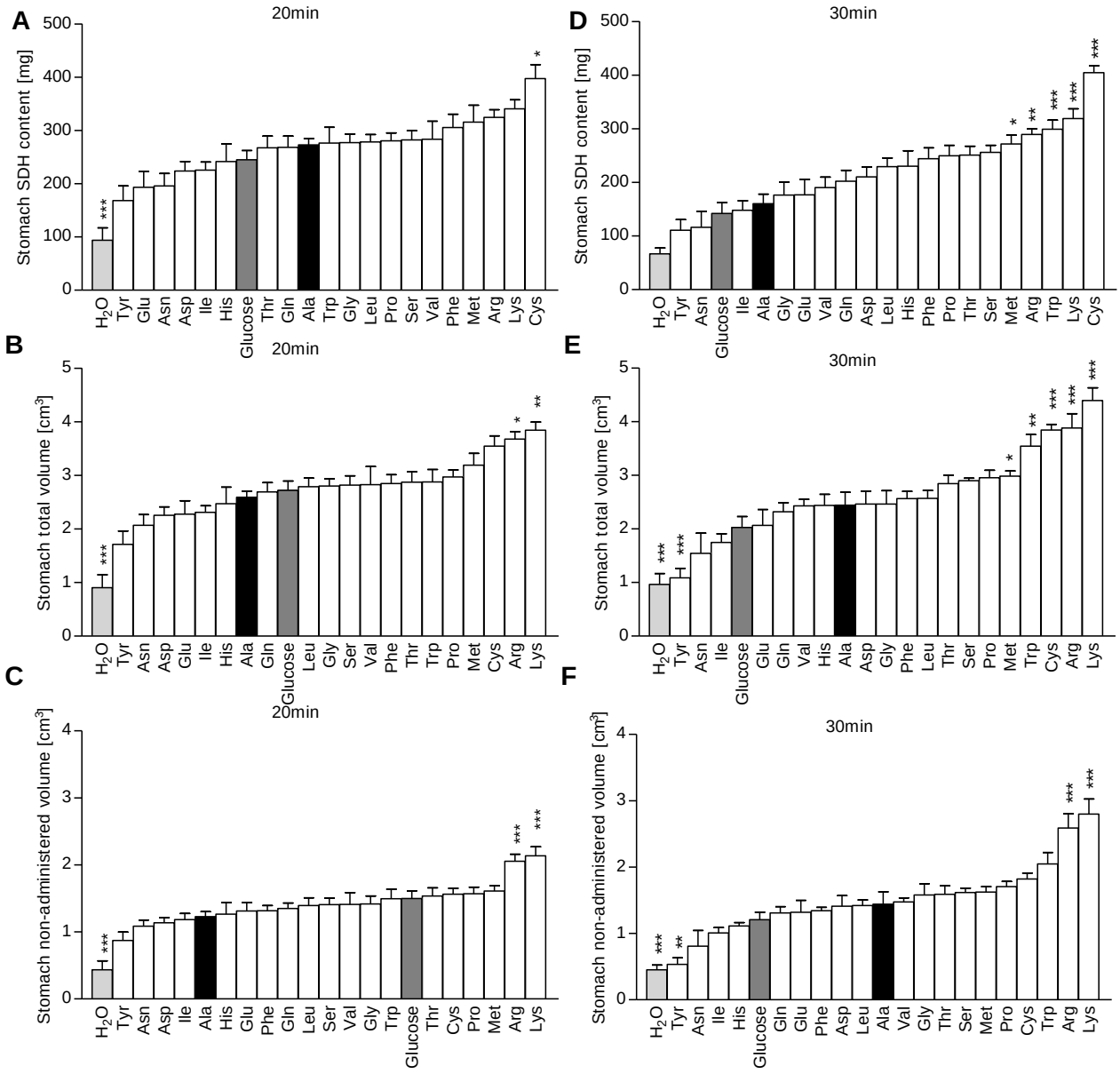


Figure 6 – Systematic study of the impact of individual amino acids on gastric function. *Fasted animals were gavaged with Sodium-diatrizoate (SDH, contrast agent) containing an individual amino acid. After 20 (A, B, C) or 30 min (D, E, F) animals were imaged using computed tomography and the contrast content in the stomach and the total stomach volume quantified. The SDH content reflects the extent of gastric emptying and the non-administered stomach volume gastric secretion. L-alanine served as a negative control and was used for statistical comparison. Values are mean ± sem; n = 11, unpaired one-way ANOVA, Dunnett Post-Test, *P<0.05, **P<0.01, ***P<0.001.*

Conclusion

This study confirmed our previous reported impact of L-arginine and L-lysine on gastric emptying and secretion by classical lethal techniques. Beyond these it identified other interesting amino acids. L-tyrosine seemed to accelerate gastric emptying and inhibit gastric secretion. L-cysteine selectively blocked gastric emptying, but did not alter gastric secretion. L-tryptophan behaved similar to L-cysteine and is particularly interesting, because it was shown to regulate CCK release most potently of all amino acids *ex vivo* (37). This study raised several questions on the underlying endocrine and vagal mechanism, which will be subject to future efforts.

8. Discussion and Outlook

My thesis builds around the hypothesis, that specific individual amino acids have a distinct impact on the control of food intake and gastric function. We tested this hypothesis by systematically assessing the individual impact of all proteogenic amino acids on food intake and gastric function; identifying their different effect strength and deciphering the mechanism of action of the most potent modulators. The two functional readouts are particularly relevant in context of the regulation of short-term nutrient intake. The amount of ingested food determines the maximal amount of ingested nutrients. Gastric function, namely emptying and secretion, dictates the concentration and the timing of nutrient release into the small intestine thereby directly impacting on plasma nutrient concentrations and their temporal appearance upon meal ingestion. Liver-, muscle metabolism, urine excretion among other functions may buffer the extent of plasma nutrient concentration changes after a meal, but they cannot directly alter absorption. In this thesis we showed a remarkable amino acid specific impact on eating behavior and gastric function. These findings are directly discussed in the respective paper and I will not repeat here these conclusions. Instead I aim to briefly discuss overall limitations of the performed studies, reveal some promising future avenues specific to each topic and finish with a general discussion about cellular amino acid sensing mechanisms.

Individual Amino Acid Administration & Dose

The main experimental intervention in my thesis was the administration of individual amino acids. They were applied orally by gavage in the two screens conducted for food intake and gastric function. Intragastric application was chosen because it follows the physiological route of a meal and the stomach is the primary place where the body is exposed to the individual amino acids contained in a meal. Correctly one can argue that the use of an individual amino acid is an artificial condition and the stomach in nature never encounters individual amino acids. Indeed a meal contains not only one but rather all the 20 proteogenic amino acids and additionally carbohydrates, fats, fibers, vitamin, salts, minerals, etc as individual molecules but also as part of higher order structure for instance membrane fragments, extracellular matrix components among others. Theoretically each of these molecules can generate a physiological signal by itself and their combination may trigger multiple potentially synergizing or neutralizing signals. To reduce the complexity of this multi nutrient matrix, we assessed the impact of only one individual amino acid on food intake and gastric function. Additionally, we showed that different feeding states and therefore the presence of other nutrients did not interfere with the individual anorectic impact of L-arginine, L-lysine or L-glutamic acid. Still, concluding that solely the three candidate amino acids mediate the anorectic effect of a high protein diet might be too simplistic. Following this argument the combination of individual amino acids i.e. L-arginine, L-lysine and L-glutamic acid should be more anorectic compared to the individual. The conducted combinatory experiments did not support this conclusion. Particularly intriguing was that L-glutamic acid inhibited the effect of L-arginine, L-lysine or their combination. Hence, the combination of different nutrients and macronutrients clearly contributes to the anorectic effect of a high protein diet. Still our data proposes a prominent role for specific individual amino acids namely L-arginine, L-lysine and L-glutamic acid.

To identify the most potent individual amino acids, we conducted our screens for food intake and gastric function at an isomolar dose of 6.7 mmol of each individual amino acid/kg body weight. Isomolar conditions were selected to enable comparison, but different alternatives exist and are briefly discussed. Naturally different combinations of the approaches are also possible, but they will not be discussed here. The first alternative is to use isocaloric doses. This approach would exclude

energetic differences between the applied doses to affect the outcome of the screens. The disadvantage is the use of different molarities and thereby comparing the effect of different numbers of chemical molecules. Isomolar conditions prevent this pitfall and were also favored by use, because the largest energy difference is 8 kJ ≈ 2 kcal between the applied doses in the isomolar screen for a 300 g rat (Table 2). This difference is very small considering the daily intake of ca. 400-500 kJ in a healthy rat (daily intake of ±28 g rat chow of KLIBA NAFAG 3430, gross energy 16.1 kJ/g). A second alternative is to assess the daily intake of individual amino acids in a healthy animal. This was done by measuring the daily food intake of a specific diet (here, 28 g of KLIBA NAFAG 3430). The amino acid content of the diet can be analytically determined (here, according to manufacturer's information) and thereby the daily intake of individual amino acids calculated (Table 2). This specific individual amino acid intake could then be applied as a single oral dose. However, several questions arise. Should the daily amino acid intake, the first meal or the intake in the first two hours of the dark phase be used as reference? What is the reference diet? Is the reference diet low or rich in proteins? What protein source is the reference – casein, peptone, albumin or another? Hence, several unknowns complicate this approach apart of the same limitations already described for the isocaloric approach above. The third alternative would be to base the dose on the circulating blood levels of individual amino acids. Here, it is unclear if fasting or postprandial levels are relevant, if portal or common circulating blood levels should be considered and if equal or multiple factors of the blood levels should be applied. The fourth alternative is to measure the changes in portal blood levels of single amino acids after the intake of a defined meal and subsequently mimicked by the application of a single amino acid dose. Naturally this approach is very laborious and the same limitations as described above are here also relevant. Overall a satisfactory compromise to include all potential factors seems to be difficult and at some point all approaches to determine a reasonable dose are arbitrary. Our aim was to compare the effect of individual amino acids against each other, hence applying the same amount of an amino acid seemed conceptually critical. Therefor we decided to use the same single isomolar dose for all amino acids of 6.7 mmol/kg. This corresponds in the case of L-glutamic acid to 1 g/kg and is in the range used by other previous publications (12, 107). Obviously, a more complete dose-response relationship for all 20 amino acid would be desirable but is not realistic from a work feasibility perspective. To consider if a dose is in a physiological range, we compared the individual doses to the daily dietary intake of an individual amino acid. As no universal standard lab diet exists and dietary protein content and source vary between labs, the comparison needs to be considered not as a fix threshold but rather as a range (Table 2).

Table 2 shows that for instance for L-glutamic acid the applied dose of 6.7 mmol/kg in a 300 g rat reflects 27 % of the normal daily L-glutamic acid intake. Most doses for individual amino acids range between 27 to 250 % of their specific daily intake. This might reflect a physiological range as for instance animals fed a high protein diet (~40 % protein) easily double their amino acid intake. The exception is L-tryptophan with a relatively high dose reflecting 729 % of the daily L-tryptophan intake, which therefore is rather a pharmacological dose. The doses are relatively similar concerning caloric or energy content and contain only 3-11 kJ. This calculation is based on the combustion energy and therefor reflects an entirely chemical measurement. Alternatively ATP equivalent or other biological energy forms could be calculated, but they are determined based on metabolic flow charts and thereby reflect as well at best an approximation. We performed correlation analysis to compare the properties of each individual amino acid dose (% daily intake, energy content, ATP equivalents, etc.) to its specific impact on food intake, gastric emptying and secretion, but no

correlation was found. In sum, we consider our approach physiological meaningful even though every dose or strategy has limitations.

Table 2 – Applied individual amino acid dose relative to the dietary intake.

Amino acid	Dietary amino acid content[1]	Amount of food intake in a 16h fasted rats[2]		Applied dose in a 300 g animal[3]		Applied dose relative to normal intake		Molar mass	Energy[4]
		Daily - 28g	1h - 8g		energy[4]	Daily	1h		
	%	mg	mg	mg	kJ	%	%	g/mol	kJ/g AA
Alanine	0.97	272	78	178	3	66	230	89.09	17.7
Arginine	***1.1***	***308***	***88***	***348***	***7***	***113***	***396***	***174.2***	***21.5***
Asparagine	-	-	-	-	-	-	-	132.12	14.6
Aspartate	1.67	468	134	266	3	57	199	133.1	12
Cystein	0.28	78	22	242	5	309	1082	121.16	18.6
Glutamate	***3.93***	***1100***	***314***	***294***	***5***	***27***	***94***	***147.13***	***15.3***
Glutamine	-	-	-	-	-	-	-	146.14	17.6
Glycine	0.84	235	67	150	2	64	223	75.07	13
Histidine	0.47	132	38	310	6	236	825	155.15	20.7
Isoleucine	0.78	218	62	262	7	120	420	131.17	27.3
Leucin	1.5	420	120	262	7	62	219	131.17	27.3
Lysine	***1***	***280***	***80***	***292***	***7***	***104***	***365***	***146.19***	***25.2***
Methionine	0.39	109	31	298	7	273	956	149.21	23
Phenylalanine	0.83	232	66	330	9	142	498	165.19	28.1
Proline	1.43	400	114	230	5	58	201	115.13	23.7
Serine	0.87	244	70	210	3	86	302	105.09	13.7
Threonine	0.65	182	52	238	4	131	458	119.12	17.2
Tryptophan	0.2	56	16	408	11	729	2553	204.23	27.6
Tyrosine	0.52	146	42	362	9	249	871	181.19	24.4
Valine	0.93	260	74	234	6	90	315	117.15	25

[1] Kliba Nafag 3430 diet

[2] see feeding data of baseline H_2O group

[3] 6.7 mmol/kg BW

[4] Combustion energy - Amino acids: metabolism, functions and nutrition; Guoyao Wu, Texas, 2009

Neuronal Activity

Feeding behavior is ultimately controlled by the brain within specific neuronal circuits. We assessed neuronal activity using immunohistochemistry to stain for the expression of the widely and commonly used early gene cFOS, which is expressed upon regulation of intracellular Ca^{2+}. Other classical but less used alternatives to cFOS are in-situ hybridization for *Arc* or *Homer 1a* gene products or immunohistochemistry against the phosphorylated form of ERK or CREB proteins (160). The advantage of cFOS is the higher stability of a protein compared to mRNA and that it does not require double labeling for the non-phosphorylated protein variant. From a methodological perspective immunohistochemistry is limited because complete brain staining is extremely laborious and therefor temporal and treatment resolution is restricted to few conditions. Additionally antibody sensitivity and specificity may differ between labs. The main disadvantage is that the correlation between neuronal activity meaning single neuronal spiking and the expression of a protein 90 min later may not be linear and it is unclear if cFOS expression is required for the observed behavioral phenotype. Nevertheless it enables to assess neuronal activity with good spatial resolution. We detected more cFOS positive cells in the area postrema and the nucleus of the solitary tract after oral L-arginine, L-lysine and L-glutamic acid administration, but not in any other brain region related to eating behavior namely the hypothalamus and the nucleus accumbens. Interestingly an acute high protein diet intake increased cFOS expression as well in the nucleus of the solitary tract but also in the arcuate nucleus of the hypothalamus reflecting a specific difference to our study (39, 45). Methodologically the papers measuring the acute effect of a high protein diet on cFOS expression are

similar to ours. We reasoned that this difference might have a biological explanation. First, a high protein diet contains carbohydrates and fats, which are absorbed, enter circulation and therefor can stimulate the glucose and fat sensitive central neurons located in the arcuate nucleus previously described in the introduction (34). Second, a high protein diet was shown to stimulate the release of gastrointestinal hormones and several of them are known to mediate their effect via arcuate neurons (167). Third, a high protein diet as well modulates body weight gain and therefor interacts with long term signals, which are thought to mainly induce their anorectic effect via the hypothalamus (103). Hence, there are several different reasons to expect different neuronal activity after the intake of a high protein meal compared to an individual amino acid.

We showed the specific importance of area postrema neurons and vagal afferents in context of the anorectic effect of L-arginine, L-lysine and L-glutamic acid by surgical lesion of the two afferent pathways. We suggest in our manuscript that L-arginine and L-glutamic acid directly act on area postrema neurons, whereas L-lysine directly on vagal afferents. This conclusion is based on the presented evidence in the manuscript and supported by the literature indicating hepatic vagal afferents recordings sensitive to L-lysine *ex vivo* (150). Direct electrophysiological recordings from area postrema neurons would be a desirable method to test, if L-arginine and L-glutamic acid but not for instance L-lysine application alters the neuronal firing rate. However, this brain region is unfavorable for electrophysiological studies due to its spongy structure caused by the strong vascularization, but still it is possible (24, 50). Alternative approaches could aim to culture area postrema neurons and perform *ex vivo* Ca^{2+} signaling under different conditions. These efforts and potential positive findings would strongly support our conclusion. Additionally they would enable to investigate the biochemical identity of neurons activated by individual amino acids and thereby on the molecular mechanism enabling neurons to distinguish between individual amino acids. Interestingly amino acids seem to be detected by two different afferent pathways indicating redundancy. To test the broader relevance of this amino acid specific finding, area postrema and vagal afferent lesion animals should be exposed to a high protein diet and their food intake recorded. Our data may be interpreted to predict that a high protein diet should lose its anorectic effect in these double-lesion animals compared to control.

Gastrointestinal Function

The study of gastrointestinal function is particularly challenging from a methodological perspective. Gastrointestinal function is fast i.e. gastric peristaltic waves have a frequency of 3 contractions per minute in humans, and the 3D convolute of the intestinal segments requires high spatial resolution for visualization. Even though several methods exist to study specifically the diverse functions, all of them are limited by throughput and do not allow to assess different gastrointestinal functions simultaneously. Currently most approaches are lethal and are very laborious apart of being limited by temporal, spatial and/or quantitative resolution. Hence, it would be desirable to develop methods enabling to assess gastric motility and secretion, pancreatic and biliary acid secretion, intestinal and colon motility, among others simultaneously. Here, we initially focused on the stomach. The novel computed tomography method is non-invasive and enables high throughput studies as demonstrated by screening for the impact of individual amino acids on gastric emptying and secretion.

The computed tomography screen outcome supported our previous data suggesting an L-arginine and L-lysine specific impact on gastric function but additionally revealed novel interesting amino acids namely L-tryptophan, L-tyrosine and L-cysteine. The previous reported effect of L-glutamic acid was as well confirmed when comparison is made to water control. The effect of L-glutamic acid is less

spectacular if compared to other amino acids but this was already apparent in the previous study (Fig. 6 and Manuscript: Specific Amino Acids Inhibit Food Intake via the Area Postrema or Vagal Afferents.). The remarkable amino acid specificity at isomolar conditions does exclude a simple osmotic effect and as well a solely calorie dependent mechanism (see small energy differences between applied doses in Table 2; max. Δ = 8 kJ ≈ 2 kcal). So far no study systematically studied the individual impact of amino acids on gastric secretion and emptying in rats. Nevertheless one study compared the effect of all individual amino acids on gastric emptying in dogs (143). The authors highlighted the effect of L-tryptophan delaying gastric emptying even though they detected as well a significant but smaller gastric emptying delay induced by L-lysine. They did not report any effect for L-arginine or L-tyrosine on gastric emptying. L-cysteine neither altered gastric emptying, but induced vomiting in dogs. Potentially these differences in amino acid specific effects arise due to species differences between rodents and dogs. This might be problematic for future translational studies as dogs are considered as the best animal model for human gastrointestinal function and may even be superior to monkeys (60). The authors of the dog study concluded that L-tryptophan may mediate its effect via CCK and noted that they surprisingly did not detect an effect after L-phenylalanine administration. Both amino acids were reported to potently induce CCK secretion and these rather classic studies were recently confirmed by novel *ex vivo* approaches (37). Therefore, it would be interesting to test if L-arginine, L-lysine, L-tryptophan, L-tyrosine or L-cysteine stimulated the release of gastrointestinal hormones. As we did not detect any hormonal concentration differences in circulation, future work should aim to disentangle their paracrine role. Hence, the effect of oral L-arginine, L-lysine, L-tryptophan, L-tyrosine or L-cysteine application on gastric secretion and emptying could be tested after the application of different gastrointestinal hormone antagonists in rats.

As gastric emptying and secretion are thought to be controlled by an intricate feedback mechanism based on vagovagal reflexes and/or gastrointestinal hormones, it may be interesting to test if secretion and emptying are controlled by the same regulatory mechanism (23). First indication could be drawn after performing dose response studies. Differences in the dose-response curve may indicate different underlying mechanisms. For L-lysine we already showed a different dose response curve for gastric emptying and secretion in rats and in humans, which may indicate independent regulatory mechanisms. Second, gastric emptying and secretion may be stimulated by signals emerging in the gastric and intestinal lumen and/or the blood stream. To improve our understanding, it is relevant to assess where amino acids induce their effect on gastric emptying and secretion. Rats could be equipped with duodenal and/or portal-vein catheters and the elicited gastric effect monitor after candidate amino acid application at both locations. All these studies may determine and improve our understanding of how L-arginine, L-lysine, L-glutamic acid, L-tryptophan, L-tyrosine and L-cysteine induce their effect on gastric emptying and secretion. They will help to identify which cell types are potentially involved and highlight the physiological important site of action.

Amino Acid Sensing

Our efforts have revealed that L-arginine and L-glutamic acid mediate their anorectic effect via area postrema neurons, whereas L-lysine by vagal afferents. Which cell type and regulatory mechanism is stimulated by L-arginine, L-lysine, L-glutamic acid, L-tryptophan, L-tyrosine or L-cysteine to modulate gastric function is under current investigation. Intuitively the identified cells are localized at a specific anatomical position to exert their function and additionally express a specific set of proteins enabling them to do so. Both studies ultimately lead to the question, what are the proteins that enable amino

acid sensing? Do they explain the basis for the observed amino acid specificity? Next I will speculate about cellular amino acid sensing mechanisms emphasizing that it is difficult to propose an "educated guess" for the underlying cellular mechanism of the here described physiological effects. One needs to consider that here probably different sensory mechanisms overlap and thereby complicate the systemic understanding of the regulatory effect induced by L-arginine, L-lysine, L-glutamic acid, L-tryptophan, L-tyrosine and L-cysteine.

A general amino acid sensor able to detect the amino group shared by all amino acids was characterized in *S. cerevisiae* highlighting the existence of unspecific amino acid sensing mechanisms. The plasma membrane permease-like protein Ssy1 stimulates the activation of the Ssy5 protease upon extracellular amino acid binding. Ssy5 cleaves Stp1 and Stp2 transcription factor N-terminally enabling their translocation into the nucleus, where they drive the expression of specific amino acid induced genes mainly amino acid transporters (91). This general sensing system is not conserved in higher eukaryotes presumably because sensing individual amino acid might be a more significant evolutionary advantage (80). The detection of individual amino acids might give a more accurate indication of protein quantity and quality intake compared to a broadly tuned amino acid sensor. This interpretation is supported by the loss of the Ssy1 mechanism in the course of evolution.

Specificity for amino acid sensing appeared as well early in evolution. Most prominent is the aspartate and arginine receptors in *E. coli*, which enables bacterial motor function ("swimming") directionality towards an amino acid rich localization (46). As these pathways do not have eukaryotic homologs they are not discussed here in further detail. Still they demonstrate the simplest mechanism for detecting the presence of a single amino acid by a "ligand receptor" interaction. An alternative strategy is to detect the lack of individual amino acids instead of their presence. Here, the GCN2 pathway plays the pivotal role as described in in the introduction. The GCN2 kinase is activated in response to binding of uncharged tRNA and was first described in *S. cerevisiae* but is also conserved in higher eukaryotes. Nevertheless functionally they differ between species. In yeast GCN2 activation stimulates the transcription of genes involved in amino acid synthesis and absorption and does not inhibit general translation. Additionally in yeast, which can synthesize all 20 amino acids, GCN2 is activated by the starvation of all individual amino acids (66). In contrast, in the rat anterior piriform cortex GCN2 activation is only induced upon starvation of essential amino acids, inhibits general translation and leads to the rejection of the specific diet (57). Hence, this evolutionary conserved amino acid sensing mechanism can detect amino acid depletion in both species, the specificity was adapted to the available anabolic pathways and importantly the induced response seems to be contrary. As lesion to the anterior piriform cortex did not abolish the anorectic effect of a high protein diet, it is very likely that the lack of an individual amino acid is sensed by a different mechanism compared to their presence (85). The challenge in sensing the presence of individual amino acid arises from their multiple functions. They are not only signal transmitters like insulin with its specific receptor family, but they are also metabolic substrates, protein building-blocks, neurotransmitters and so on. Due to this multi-functionality amino acids may be sensed by several most probably redundant mechanisms making identification difficult. Next, the currently available concepts will be briefly discussed starting with the simple receptor – ligand interaction, transceptors and finishing with intracellular nutrient kinases. A focus to amino acids is given in particular to the ones inducing either a gastric or anorectic effect in the here presented thesis.

The most prominent receptors directly responding to amino acids in mammals are several G-protein couple receptors (128). The Ca^{2+} - sensing receptor (CaSR) does not only sense Ca^{2+} but also several

amino acids among other molecules (132). In culture L-phenylalanine and L-tryptophan mobilized Ca^{2+} most potently compared to all other amino acids in the presence of Ca^{2+}. Generally charged amino acids were much less effective compared to aromatic amino acids and nearly no effect had branch chain amino acids (31). CaSR knockout mice died early after birth demonstrating the general importance of this receptor (163). Endothelia specific CaSR-knockout animals did not show differences in eating behavior or body weight gain (personal communication – Pascal Houillier). The CaSR is strongly expressed in the area postrema suggesting a potential role in feeding behavior, but this was so far not specifically tested (47). The high expression in the area postrema could as well be linked to other functions of the area postrema, particularly in the context of sodium, potassium or calcium homeostasis. For instance it was shown that AP lesion animals have a supernormal desire for NaCl intake without apparent impact on NaCl loss (33). The low affinity for L-arginine, L-lysine and L-glutamic acid suggests no role of the CaSR in the control of their anorectic effect due to differences in amino acid specificity. In context of gastrointestinal function the CaSR is expressed in several endocrine cells along the gastrointestinal tract and associated with their secretory functions (30, 54). For instance CCK-secretion from murine intestinal I cells was shown to depend on the CaSR and was most potently induced by L-tryptophan (90). Additionally gastrin and somatostatin co-localize with the CaSR indicating another potential secretory role (61). In summary, the CaSR is relevant for chemosensing in the gut and might be relevant for the delay of gastric emptying observed after L-tryptophan administration. An evolutionary neighbor of the CaSR is the G-protein coupled receptor group 6 family A (GPRC6A) protein. GPRC6A is an osteocalcin receptor which is stimulated by basic amino acids particularly L-arginine, L-lysine and L-ornithine (162). The receptor is broadly expressed including in the brain but no study so far addressed its expression in the area postrema specifically (161). Interestingly knockout animals of GPRC6A have osteopenia, feminization and metabolic syndrome. They show reduced body weight, increased body fat and glucose intolerance (120). Based on the broad functional impact, the authors suggest that GPRC6A may coordinate the anabolic response of multiple tissues to amino acids. In the gastric tract GPRC6A was shown to co-localize with gastrin and somatostatin (61). Hence, it might be tempting to speculate that GPRC6A mediates some of the effects induced by L-arginine and L-lysine. Another receptor, the T1R1/T1R3, might be another interesting candidate for amino acid detection as discussed in the introduction. Taken together several G-protein couple receptors respond to individual amino acids. They were shown to play a prominent role for different endocrine functions, but their role in mediating neuronal firing is unclear. Nevertheless we do not expect them to mediate the anorectic effect of L-arginine, L-lysine and L-glutamic acid because of their broad specificity. Concerning the gastric effect observed, it might be that L-arginine, L-lysine, L-tryptophan, L-tyrosine and L-cysteine mediate their effect indirectly by stimulating endocrine cells to release gastrointestinal hormones by one of the above or a still unknown receptor. One could test for the involvement of a receptor by administering amino acids coupled to polyethylenglycol, which renders amino acid still accessible but not absorbable. Conceptually a receptor should be able to bind this form similar to the non-modified amino acid and induce its downstream effect.

Intestinal enterocytes and endocrine cells express a large variety of amino acid transporters at the apical surface and presumably area postrema neurons do so as well. The precise expression pattern is most probably cell type specific and thereby may reveal interesting differences. The transporters ensure efficient amino acid absorption thereby altering the specific intracellular amino acid pool relevant for intracellular amino acid sensing mechanisms (discussed below). The newly emerging concept of “transceptors” suggests, that transporters not only shuttle specific nutrients but are as

well able to signal specifically relatively to the transported nutrient (69, 99). Secondary active transport couples amino acid transport with the inward movement of for instance sodium following the electrochemical gradient. The induced ionic current could trigger voltage sensitive signaling mechanism. For instance the release of GLP1 was shown to be triggered by membrane depolarization caused by calcium entry after sodium dependent glutamine uptake (127, 129). However, so far knockout experiments for the voltage dependent calcium influx transporters are missing to show the *in vivo* relevance of this mechanism. Other gradients such as potassium or even tertiary transport could be coupled to alter membrane potential and thereby alter intracellular calcium stores. Clearly the difficulty of this concept is to separate transport from signaling function. Unfortunately the "transcepter" concept does not clarify how to interpret the overlapping substrate specificity of several amino acid transporters, the interaction of different transport proteins with distinct accessory proteins, the different role of the apical and the basolateral membrane and the potential importance of intracellular membranes. From the perspective of L-arginine, L-lysine, L-glutamic acid, L-tryptophan, L-tyrosine or L-cysteine "transcepting" the number of potential transporters is large. To formulate a hypothesis for the involved transport proteins it would be clearly beneficial to know their expression profile in the area postrema respectively of vagal afferent fibers and ideally from the cells activated by the candidate amino acids.

The most prominent intracellular nutrient sensing/signaling mechanism is the mTORC1 pathway as briefly mentioned in the introduction. mTORC1 not only regulates protein but also lipid synthesis which are both coupled to the availability of the respective nutrient (133). Amino acid limitation inhibits protein synthesis and induces autophagy via the mTORC1 kinase, presumably to maintain intracellular amino acid pools (14, 115). The most potent inducer of mTORC1 signaling are the amino acids L-arginine and L-leucine in different cell culture system, but there impact is limited if all other amino acids are depleted (63). This might arise as they are not efficiently transported in the absence of other amino acids. For instance, L-leucine induced mTORC1 signaling was shown to be driven by the anti-port of intracellular glutamine for extracellular L-leucine (108). What the upstream mechanism is to detect L-leucine and L-arginine is part of current research effort and therefore the selectivity to L-arginine and L-leucine is not well understood (80). mTORC1 is ubiquitously expressed in the rat brain including the nucleus of the solitary tract (35). Injection of L-leucine into the nucleus of the solitary tract did not induce phosphorylation of mTOR in the area postrema (15). Unfortunately we never tested for mTORC1 activation after L-arginine, L-lysine or L-glutamic acid administration in the area postrema or the nucleus of the solitary tract by immunohistochemistry or by other means. A definite role of mTORC1 could have been validated by administration of rapamycin intravenously as a specific mTOR inhibitor. Overall mTORC1 is the best characterized intracellular nutrient sensing pathway and potentially an interesting future avenue.

Our efforts revealed specific physiological effects of L-arginine, L-lysine, L-glutamic acid, L-tryptophan, L-tyrosine and L-cysteine on animal behavior and also on gastrointestinal function. The main question arising of all the studies is which cellular mechanism enables to mediate this remarkable amino acid specificity. Different potential candidate proteins were suggested above and a future safe avenue would be to test specifically for their involvement. The main argument against all the candidate mechanism is that it is unclear how they should mediate the specificity to the individual amino acids. Here attention has to be taken – neither the screen for the impact of individual amino acids on food intake nor on gastric function has revealed a binary result. Only statistical analysis based on multi-condition comparison suggests this outcome, but reality suggests a certain continuity of responses to individual amino acids. Hence, it might be that one of the above

receptors is involved in mediating some of the here reported *in vivo* functions even though there is a non-perfect receptor specificity match. In the long term it would be desirable to establish a system, where we could specifically test for the role of candidate proteins respectively systematical screen the rat genome. *Ex vivo* cultures of area postrema neurons combined with Ca^{2+} imaging might provide such a system for systemically probing the regulation of neuronal activity in the near future. Still mayor logistic and methodological hurdles need to be taken before genome wide screening is possible. In context of the regulation of gastrointestinal function it is still unclear, which cell types are relevant for the response. Most probably the effects are a systemic and therefor finding a suitable model for systematic testing is a formidable challenge. In sum, the identification of the molecular machinery responsible for the here reported physiological effects induced by individual amino acids is a fascinating and also an ultimate challenge not only in respect of the current available technology.

9. References

1. **Abbott, C. R., M. Monteiro, C. J. Small, A. Sajedi, K. L. Smith, J. R. C. Parkinson, M. A. Ghatei, and S. R. Bloom.** 2005. The inhibitory effects of peripheral administration of peptide YY3–36 and glucagon-like peptide-1 on food intake are attenuated by ablation of the vagal–brainstem–hypothalamic pathway. Brain Research **1044**:127–131.
2. **Allen, T., F. Zhang, S. A. Moodie, L. E. Clemens, A. Smith, F. Gregoire, A. Bell, G. E. Muscat, and T. A. Gustafson.** 2006. Halofenate Is a Selective Peroxisome Proliferator–Activated Receptor γ Modulator With Antidiabetic Activity. Diabetes **55**:2523–2533.
3. **Anderson, G. H., and S. E. Moore.** 2004. Dietary proteins in the regulation of food intake and body weight in humans. J Nutr **134**:974S-9S.
4. **Anderson, G. H., S. Luo, L. T. Ng, and E. T. S. Li.** 1994. Non-essential amino acids and short-term food intake of rats. Nutrition Research **14**:1179–1189.
5. **Anderson, G. H., S. Luo, L. Trigazis, G. Kubis, and E. T. Li.** 1994. Effects of essential amino acids on food and water intake of rats. Can J Physiol Pharmacol **72**:841–848.
6. **Anderson, G. H., S. N. Tecimer, D. Shah, and T. A. Zafar.** 2004. Protein Source, Quantity, and Time of Consumption Determine the Effect of Proteins on Short-Term Food Intake in Young Men. The Journal of Nutrition **134**:3011–3015.
7. **B.D. White, R.G Dean, and R.J Martin.** 1998. An association between low levels of dietary protein, elevated NPY gene expression in the basomedial hypothalamus and increased food intake. Nutr Neurosci:173–182.
8. **Badman, M. K., and J. S. Flier.** 2005. The Gut and Energy Balance: Visceral Allies in the Obesity Wars. Science **307**:1909–1914.
9. **Batterham, R. L., H. Heffron, S. Kapoor, J. E. Chivers, K. Chandarana, H. Herzog, C. W. Le Roux, E. L. Thomas, J. D. Bell, and D. J. Withers.** 2006. Critical role for peptide YY in protein-mediated satiation and body-weight regulation. Cell Metab **4**:223–233.
10. **Bensaïd, A., D. Tomé, D. L'Heureux-Bourdon, P. Even, D. Gietzen, C. Morens, C. Gaudichon, C. Larue-Achagiotis, and G. Fromentin.** 2003. A high-protein diet enhances satiety without conditioned taste aversion in the rat. Physiology & Behavior **78**:311–320.
11. **Berthoud, H.-R.** 2002. Multiple neural systems controlling food intake and body weight. Neurosci Biobehav Rev **26**:393–428.
12. **Bialik, R. J., E. T. Li, P. Geoffroy, and G. H. Anderson.** 1989. Route of delivery of phenylalanine influences its effect on short-term food intake in adult male rats. J Nutr **119**:1519–1527.
13. **Blom, W., A. Lluch, A. Stafleu, S. Vinoy, J. J. Holst, G. Schaafsma, and H. F. J. Hendriks.** 2006. Effect of a high-protein breakfast on the postprandial ghrelin response. The American Journal of Clinical Nutrition **83**:211–220.
14. **Blommaart, E. F. C., J. J. F. P. Luiken, P. J. E. Blommaart, G. M. van Woerkom, and A. J. Meijer.** 1995. Phosphorylation of Ribosomal Protein S6 Is Inhibitory for Autophagy in Isolated Rat Hepatocytes. Journal of Biological Chemistry **270**:2320–2326.
15. **Blouet, C., and G. J. Schwartz.** 2012. Brainstem Nutrient Sensing in the Nucleus of the Solitary Tract Inhibits Feeding. Cell Metab.
16. **Boey, D., S. Lin, T. Karl, P. Baldock, N. Lee, R. Enriquez, M. Couzens, K. Slack, R. Dallmann, A. Sainsbury, and H. Herzog.** 2006. Peptide YY ablation in mice leads to the development of hyperinsulinaemia and obesity. Diabetologia **49**:1360–1370.
17. **Bostrom, P., J. Wu, M. P. Jedrychowski, A. Korde, L. Ye, J. C. Lo, K. A. Rasbach, E. A. Bostrom, J. H. Choi, J. Z. Long, S. Kajimura, M. C. Zingaretti, B. F. Vind, H. Tu, S. Cinti, K. Hojlund, S. P. Gygi, and B. M. Spiegelman.** 2012. A PGC1-[agr]-dependent myokine that drives brown-fat-like development of white fat and thermogenesis. Nature **481**:463–468.
18. **Boutry, C., C. Bos, H. Matsumoto, P. Even, D. Azzout-Marniche, D. Tome, and F. Blachier.** 2011. Effects of monosodium glutamate supplementation on glutamine metabolism in adult rats. Front Biosci (Elite Ed) **3**:279–290.

19. **Bowen, J., M. Noakes, and P. M. Clifton.** 2006. Appetite regulatory hormone responses to various dietary proteins differ by body mass index status despite similar reductions in ad libitum energy intake. J Clin Endocrinol Metab **91**:2913–2919.
20. **Bowen, J., M. Noakes, C. Trenerry, and P. M. Clifton.** 2006. Energy intake, ghrelin, and cholecystokinin after different carbohydrate and protein preloads in overweight men. J Clin Endocrinol Metab **91**:1477–1483.
21. **Brener, W., T. R. Hendrix, and P. R. McHugh.** 1983. Regulation of the gastric emptying of glucose. Gastroenterology **85**:76–82.
22. **C W Howden, and R H Hunt.** 1987. Relationship between gastric secretion and infection. Gut:96–107.
23. **Camilleri, M.** 2006. Integrated upper gastrointestinal response to food intake. Gastroenterology **131**:640–658.
24. **Cechetto, D. F., T. Diab, C. J. Gibson, and A. W. Gelb.** 2001. The Effects of Propofol in the Area Postrema of Rats. Anesthesia & Analgesia **92**:934–942.
25. **Choi, Y. H., N. Chang, and G. H. Anderson.** 1999. An intragastric amino acid mixture influences extracellular amino acid profiles in the lateral hypothalamic area of freely moving rats. Can J Physiol Pharmacol **77**:827–834.
26. **Choi, Y. H., N. Chang, P. J. Fletcher, and G. H. Anderson.** 2000. Dietary protein content affects the profiles of extracellular amino acids in the medial preoptic area of freely moving rats. Life Sci **66**:1105–1118.
27. **Choi, Y. H., P. J. Fletcher, and G. H. Anderson.** 2001. Extracellular amino acid profiles in the paraventricular nucleus of the rat hypothalamus are influenced by diet composition. Brain Res **892**:320–328.
28. **Choi, Y. H., P. J. Fletcher, C. C. Wong, and G. H. Anderson.** 1999. Measurement of blood-brain barrier permeability of rats with alpha-aminoisobutyric acid during microdialysis: possible application to behavioral studies. Physiol Behav **67**:587–598.
29. **Collier, G., A. I. Leshner, and R. L. Squibb.** 1969. Dietary self-selection in active and non-active rats. Physiology & Behavior **4**:79–82.
30. **Conigrave, A. D., and E. M. Brown.** 2006. Taste Receptors in the Gastrointestinal Tract II. l-Amino acid sensing by calcium-sensing receptors: implications for GI physiology. American Journal of Physiology - Gastrointestinal and Liver Physiology **291**:G753-G761.
31. **Conigrave, A. D., S. J. Quinn, and E. M. Brown.** 2000. l-Amino acid sensing by the extracellular Ca2+-sensing receptor. Proceedings of the National Academy of Sciences **97**:4814–4819.
32. **Conigrave, A. D., S. J. Quinn, and E. M. Brown.** 2000. L-amino acid sensing by the extracellular Ca2+-sensing receptor. Proc Natl Acad Sci U S A **97**:4814–4819.
33. **Contreras, R. J., and P. W. Stetson.** 1981. Changes in salt intake after lesions of the area postrema and the nucleus of the solitary tract in rats. Brain Research **211**:355–366.
34. **Cota, D., K. Proulx, and R. J. Seeley.** 2007. The role of CNS fuel sensing in energy and glucose regulation. Gastroenterology **132**:2158–2168.
35. **Cota, D., K. Proulx, K. A. B. Smith, S. C. Kozma, G. Thomas, S. C. Woods, and R. J. Seeley.** 2006. Hypothalamic mTOR Signaling Regulates Food Intake. Science **312**:927–930.
36. **Curtis, K. J., Y. S. Kim, J. M. Perdomo, D. B. Silk, and J. S. Whitehead.** 1978. Protein digestion and absorption in the rat. J Physiol **274**:409–419.
37. **Daly, K., M. Al-Rammahi, A. W. Moran, M. Marcello, Y. Ninomiya, and S. P. Shirazi-Beechey.** 2012. Sensing of amino acids by the gut-expressed taste receptor, T1R1-T1R3, stimulates CCK secretion. American Journal of Physiology - Gastrointestinal and Liver Physiology.
38. **Damak, S., M. Rong, K. Yasumatsu, Z. Kokrashvili, V. Varadarajan, S. Zou, P. Jiang, Y. Ninomiya, and R. F. Margolskee.** 2003. Detection of Sweet and Umami Taste in the Absence of Taste Receptor T1r3. Science **301**:850–853.
39. **Darcel, N., G. Fromentin, H. E. Raybould, S. Gougis, D. W. Gietzen, and D. Tomé.** 2005. Fos-Positive Neurons Are Increased in the Nucleus of the Solitary Tract and Decreased in the

Ventromedial Hypothalamus and Amygdala by a High-Protein Diet in Rats. The Journal of Nutrition **135**:1486–1490.

40. **Davis, J. D., G. P. Smith, and J. Miesner.** 1993. Postpyloric stimuli are necessary for the normal control of meal size in real feeding and sham feeding rats. Am J Physiol **265**:R888-95.
41. **Dibattista, D., and M. D. Holder.** 1998. Enhanced preference for a protein-containing diet in response to dietary protein restriction. Appetite **30**:237–254.
42. **Dixon, J. B., C. W. Le Roux, F. Rubino, and P. Zimmet.** 2012. Bariatric surgery for type 2 diabetes. Lancet **379**:2300–2311.
43. **Du, F., D. A. Higginbotham, and B. D. White.** 2000. Food intake, energy balance and serum leptin concentrations in rats fed low-protein diets. J Nutr **130**:514–521.
44. **Faipoux, R., D. Tome, A. Bensaid, C. Morens, E. Oriol, L. M. Bonnano, and G. Fromentin.** 2006. Yeast proteins enhance satiety in rats. J Nutr **136**:2350–2356.
45. **Faipoux, R., D. Tomé, S. Gougis, N. Darcel, and G. Fromentin.** 2008. Proteins Activate Satiety-Related Neuronal Pathways in the Brainstem and Hypothalamus of Rats. The Journal of Nutrition **138**:1172–1178.
46. **Falke, J. J., R. B. Bass, S. L. Butler, S. A. Chervitz, and M. A. Danielson.** 1997. THE TWO-COMPONENT SIGNALING PATHWAY OF BACTERIAL CHEMOTAXIS: A Molecular View of Signal Transduction by Receptors, Kinases, and Adaptation Enzymes. Annu. Rev. Cell Dev. Biol **13**:457–512.
47. **Ferry, S., E. Traiffort, J. Stinnakre, and M. Ruat.** 2000. Developmental and adult expression of rat calcium-sensing receptor transcripts in neurons and oligodendrocytes. European Journal of Neuroscience **12**:872–884.
48. **Fraser, K. A., E. Raizada, and J. S. Davison.** 1995. Oral-pharyngeal-esophageal and gastric cues contribute to meal-induced c-fos expression. Am J Physiol **268**:R223-30.
49. **Fromentin, G., and S. Nicolaidis.** 1996. Rebalancing essential amino acids intake by self-selection in the rat. Br J Nutr **75**:669–682.
50. **Fry, M., P. M. Smith, T. D. Hoyda, M. Duncan, R. S. Ahima, K. A. Sharkey, and A. V. Ferguson.** 2006. Area Postrema Neurons Are Modulated by the Adipocyte Hormone Adiponectin. The Journal of Neuroscience **26**:9695–9702.
51. **Fu, W. J., T. E. Haynes, R. Kohli, J. Hu, W. Shi, T. E. Spencer, R. J. Carroll, C. J. Meininger, and G. Wu.** 2005. Dietary L-arginine supplementation reduces fat mass in Zucker diabetic fatty rats. J Nutr **135**:714–721.
52. **Garlicki, J., P. K. Konturek, J. Majka, N. Kwiecien, and S. J. Konturek.** 1990. Cholecystokinin receptors and vagal nerves in control of food intake in rats. Am J Physiol **258**:E40-5.
53. **Geary, N.** 2013. Understanding synergy. American Journal of Physiology - Endocrinology And Metabolism **304**:E237-E253.
54. **Geibel, J. P., and S. C. Hebert.** 2009. The Functions and Roles of the Extracellular Ca2+–Sensing Receptor along the Gastrointestinal Tract. Annual Review of Physiology **71**:205–217.
55. **Geraedts, M. C. P., F. J. Troost, R. Tinnemans, J. D. Soderholm, R.-J. Brummer, and W. H. M. Saris.** 2010. Release of satiety hormones in response to specific dietary proteins is different between human and murine small intestinal mucosa. Ann Nutr Metab **56**:308–313.
56. **Ghosh, T., D. I. Lewis, A. T. R. Axon, and S. M. Everett.** 2011. Review article: methods of measuring gastric acid secretion. Aliment Pharmacol Ther **33**:768–781.
57. **Gietzen, D. W., S. Hao, and T. G. Anthony.** 2007. Mechanisms of food intake repression in indispensable amino acid deficiency. Annu Rev Nutr **27**:63–78.
58. **Goetze, O., A. Steingoetter, D. Menne, I. R. van der Voort, M. A. Kwiatek, P. Boesiger, D. Weishaupt, M. Thumshirn, M. Fried, and W. Schwizer.** 2007. The effect of macronutrients on gastric volume responses and gastric emptying in humans: A magnetic resonance imaging study. Am J Physiol Gastrointest Liver Physiol **292**:G11-7.
59. **Gosby, A. K., A. D. Conigrave, N. S. Lau, M. A. Iglesias, R. M. Hall, S. A. Jebb, J. Brand-Miller, I. D. Caterson, D. Raubenheimer, and S. J. Simpson.** 2011. Testing protein leverage in lean humans: a randomised controlled experimental study. PLoS One **6**:e25929.

60. **Greaves, P., A. Williams, and M. Eve.** 2004. First dose of potential new medicines to humans: how animals help. Nat Rev Drug Discov **3**:226–236.
61. **Haid, D., P. Widmayer, and H. Breer.** 2011. Nutrient sensing receptors in gastric endocrine cells. J Mol Hist **42**:355-364.
62. **Hao, S., J. W. Sharp, C. M. Ross-Inta, B. J. McDaniel, T. G. Anthony, R. C. Wek, D. R. Cavener, B. C. McGrath, J. B. Rudell, T. J. Koehnle, and D. W. Gietzen.** 2005. Uncharged tRNA and sensing of amino acid deficiency in mammalian piriform cortex. Science **307**:1776–1778.
63. **Hara, K., K. Yonezawa, Q.-P. Weng, M. T. Kozlowski, C. Belham, and J. Avruch.** 1998. Amino Acid Sufficiency and mTOR Regulate p70 S6 Kinase and eIF-4E BP1 through a Common Effector Mechanism. Journal of Biological Chemistry **273**:14484–14494.
64. **Hargrave, S. L., and K. P. Kinzig.** 2012. Repeated gastric distension alters food intake and neuroendocrine profiles in rats. Physiol Behav **105**:975–981.
65. **Hewson-Hughes, A. K., V. L. Hewson-Hughes, A. T. Miller, S. R. Hall, S. J. Simpson, and D. Raubenheimer.** 2011. Geometric analysis of macronutrient selection in the adult domestic cat, Felis catus. J Exp Biol **214**:1039–1051.
66. **Hinnebusch, A. G.** 2005. TRANSLATIONAL REGULATION OF GCN4 AND THE GENERAL AMINO ACID CONTROL OF YEAST*. Annu. Rev. Microbiol **59**:407–450.
67. **Horowitz, M., M. A. L. Edelbroek, J. M. Wishart, and J. W. Straathof.** 1993. Relationship between oral glucose tolerance and gastric emptying in normal healthy subjects. Diabetologia **36**:857-862.
68. **Huang, X., D. P. Hancock, A. K. Gosby, A. C. McMahon, S. M. C. Solon, D. G. Le Couteur, A. D. Conigrave, D. Raubenheimer, and S. J. Simpson.** 2013. Effects of dietary protein to carbohydrate balance on energy intake, fat storage, and heat production in mice. Obesity (Silver Spring) **21**:85–92.
69. **Hundal, H. S., and P. M. Taylor.** 2009. Amino acid transceptors: gate keepers of nutrient exchange and regulators of nutrient signaling. American Journal of Physiology - Endocrinology And Metabolism **296**:E603-E613.
70. **Hunt, J. N., J. L. Smith, and C. L. Jiang.** 1985. Effect of meal volume and energy density on the gastric emptying of carbohydrates. Gastroenterology **89**:1326–1330.
71. **Huo, L., L. Maeng, C. Bjørbæk, and H. J. Grill.** 2007. Leptin and the Control of Food Intake: Neurons in the Nucleus of the Solitary Tract Are Activated by Both Gastric Distension and Leptin. Endocrinology **148**:2189–2197.
72. **J Schirra, M. K. C. W., T Schäfer, U Wank, R Arnold, and B Göke.** 1996. Gastric emptying and release of incretin hormones after glucose ingestion in humans. J Clin Invest:92–103.
73. **Janssen, P., P. Vanden Berghe, S. Verschueren, A. Lehmann, I. Depoortere, and J. Tack.** 2011. Review article: the role of gastric motility in the control of food intake. Alimentary Pharmacology & Therapeutics **33**:880–894.
74. **Jean, C., G. Fromentin, D. Tome, and C. Larue-Achagiotis.** 2002. Wistar rats allowed to self-select macronutrients from weaning to maturity choose a high-protein, high-lipid diet. Physiol Behav **76**:65–73.
75. **Jean, C., S. Rome, V. Mathe, J. F. Huneau, N. Aattouri, G. Fromentin, C. L. Achagiotis, and D. Tome.** 2001. Metabolic evidence for adaptation to a high protein diet in rats. J Nutr **131**:91–98.
76. **Karnani, M. M., J. Apergis-Schoute, A. Adamantidis, L. T. Jensen, L. de Lecea, L. Fugger, and D. Burdakov.** 2011. Activation of central orexin/hypocretin neurons by dietary amino acids. Neuron **72**:616–629.
77. **Keller, U.** 2011. Proteins in human nutrition: review and recommendations of the Swiss Federal Commission for Nutrition (FCN) 2011. Int J Vitam Nutr Res **81**:85.
78. **Kitamura, A., W. Sato, H. Uneyama, K. Torii, and A. Niijima.** 2011. Effects of intragastric infusion of inosine monophosphate and L: -glutamate on vagal gastric afferent activity and subsequent autonomic reflexes. J Physiol Sci **61**:65–71.
79. **Kyriazakis, I., and G. C. Emmans.** 1991. Diet selection in pigs: dietary choices made by growing pigs following a period of underfeeding with protein. Animal Science **52**:337–346.

80. **Lamb, R. F.** 2012. Amino acid sensing mechanisms: an Achilles heel in cancer? FEBS Journal **279**:2624–2631.
81. **Laviano, A., M. M. Meguid, A. Inui, and F. Rossi-Fanelli.** 2006. Role of leucine in regulating food intake. Science **313**:1236-8; author reply 1236-8.
82. **Leathwood, P. D., and D. V. Ashley.** 1983. Strategies of protein selection by weanling and adult rats. Appetite **4**:97–112.
83. **Lejeune, M. P. G. M., K. R. Westerterp, T. C. M. Adam, N. D. Luscombe-Marsh, and M. S. Westerterp-Plantenga.** 2006. Ghrelin and glucagon-like peptide 1 concentrations, 24-h satiety, and energy and substrate metabolism during a high-protein diet and measured in a respiration chamber. Am J Clin Nutr **83**:89–94.
84. **Leshner, A. I., G. H. Collier, and R. L. Squibb.** 1971. Dietary self-selection at cold temperatures. Physiol Behav **6**:1–3.
85. **Leung, P. M., and Q. R. Rogers.** 1971. Importance of prepyriform cortex in food-intake response of rats to amino acids. Am J Physiol **221**:929–935.
86. **Leung, P. M.-B., and Q. R. Rogers.** 1969. Food intake: Regulation by plasma amino acid pattern. Life Sciences **8**:1–9.
87. **L'Heureux-Bouron, D., D. Tome, A. Bensaid, C. Morens, C. Gaudichon, and G. Fromentin.** 2004. A very high 70%-protein diet does not induce conditioned taste aversion in rats. J Nutr **134**:1512–1515.
88. **L'Heureux-Bouron, D., D. Tome, A. Bensaid, C. Morens, M. Lacroix, J. F. Huneau, and G. Fromentin.** 2004. Preabsorptive factors are not the main determinants of intake depression induced by a high-protein diet in the rat. Physiol Behav **81**:499–504.
89. **L'Heureux-Bouron, D., D. Tome, O. Rampin, P. C. Even, C. Larue-Achagiotis, and G. Fromentin.** 2003. Total subdiaphragmatic vagotomy does not suppress high protein diet-induced food intake depression in rats. J Nutr **133**:2639–2642.
90. **Liou, A. P., Y. Sei, X. Zhao, J. Feng, X. Lu, C. Thomas, S. Pechhold, H. E. Raybould, and S. A. Wank.** 2011. The extracellular calcium-sensing receptor is required for cholecystokinin secretion in response to l-phenylalanine in acutely isolated intestinal I cells. American Journal of Physiology - Gastrointestinal and Liver Physiology **300**:G538-G546.
91. **Ljungdahl PO.** Amino-acid-induced signalling via the SPS-sensing pathway in yeast. Biochem Soc Trans **2009**:242–247.
92. **Marmonier, C., D. Chapelot, and J. Louis-Sylvestre.** 2000. Effects of macronutrient content and energy density of snacks consumed in a satiety state on the onset of the next meal. Appetite **34**:161–168.
93. **Marmonier, C., D. Chapelot, M. Fantino, and J. Louis-Sylvestre.** 2002. Snacks consumed in a nonhungry state have poor satiating efficiency: influence of snack composition on substrate utilization and hunger. Am J Clin Nutr **76**:518–528.
94. **Martinez-Cordero, C., C. W. Kuzawa, D. M. Sloboda, J. Stewart, S. J. Simpson, and D. Raubenheimer.** 2012. Testing the Protein Leverage Hypothesis in a free-living human population. Appetite **59**:312–315.
95. **McArthur, K. E., J. I. Isenberg, D. L. Hogan, and S. J. Dreier.** 1983. Intravenous infusion of L-isomers of phenylalanine and tryptophan stimulate gastric acid secretion at physiologic plasma concentrations in normal subjects and after parietal cell vagotomy. J Clin Invest **71**:1254–1262.
96. **McCann, M. J., and E. M. Stricker.** 1986. Gastric emptying of glucose loads in rats: effects of insulin-induced hypoglycemia. Am J Physiol **251**:R609-13.
97. **McColl, K. E. L., E. El-Omar, and D. Gillen.** 1998. Interactions between H. pylori infection, gastric acid secretion and anti-secretory therapy. British Medical Bulletin **54**:121–138.
98. **McKnight, J. R., M. C. Satterfield, W. S. Jobgen, S. B. Smith, T. E. Spencer, C. J. Meininger, C. J. McNeal, and G. Wu.** 2010. Beneficial effects of L-arginine on reducing obesity: potential mechanisms and important implications for human health. Amino Acids **39**:349–357.
99. **Miguel-Aliaga, I.** 2012. Nerveless and gutsy: intestinal nutrient sensing from invertebrates to humans. Nutrient Sensing Pancreas Development **23**:614–620.

100. **Miller, M. G., and J. F. Teates.** 1985. Acquisition of dietary self-selection in rats with normal and impaired oral sensation. Physiol Behav **34**:401–408.
101. **Moran, L. J., N. D. Luscombe-Marsh, M. Noakes, G. A. Wittert, J. B. Keogh, and P. M. Clifton.** 2005. The satiating effect of dietary protein is unrelated to postprandial ghrelin secretion. J Clin Endocrinol Metab **90**:5205–5211.
102. **Morrison, C. D., S. D. Reed, and T. M. Henagan.** 2012. Homeostatic regulation of protein intake: in search of a mechanism. Am J Physiol Regul Integr Comp Physiol **302**:R917-28.
103. **Morton, G. J., D. E. Cummings, D. G. Baskin, G. S. Barsh, and M. W. Schwartz.** 2006. Central nervous system control of food intake and body weight. Nature **443**:289–295.
104. **Morton, G. J., D. E. Cummings, D. G. Baskin, G. S. Barsh, and M. W. Schwartz.** 2006. Central nervous system control of food intake and body weight. Nature **443**:289–295.
105. **Nassl, A.-M., I. Rubio-Aliaga, M. Sailer, and H. Daniel.** 2011. The intestinal peptide transporter PEPT1 is involved in food intake regulation in mice fed a high-protein diet. PLoS One **6**:e26407.
106. **Nelson, G., J. Chandrashekar, M. A. Hoon, L. Feng, G. Zhao, N. J. P. Ryba, and C. S. Zuker.** 2002. An amino-acid taste receptor. Nature **416**:199–202.
107. **Ng, L. T., and G. H. Anderson.** 1992. Route of administration of tryptophan and tyrosine affects short-term food intake and plasma and brain amino acid concentrations in rats. J Nutr **122**:283–293.
108. **Nicklin, P., P. Bergman, B. Zhang, E. Triantafellow, H. Wang, B. Nyfeler, H. Yang, M. Hild, C. Kung, C. Wilson, V. E. Myer, J. P. MacKeigan, J. A. Porter, Y. K. Wang, L. C. Cantley, P. M. Finan, and L. O. Murphy.** 2009. Bidirectional Transport of Amino Acids Regulates mTOR and Autophagy. Cell **136**:521–534.
109. **Niijima, A.** 1991. Effect of umami taste stimulations on vagal efferent activity in the rat. Brain Res Bull **27**:393–396.
110. **Niijima, A.** 2000. Reflex effects of oral, gastrointestinal and hepatoportal glutamate sensors on vagal nerve activity. J Nutr **130**:971S-3S.
111. **Niijima, A., and M. M. Meguid.** 1995. An electrophysiological study on amino acid sensors in the hepato-portal system in the rat. Obes Res **3 Suppl 5**:741S-745S.
112. **Norgren, R., and G. P. Smith.** 1988. Central distribution of subdiaphragmatic vagal branches in the rat. J. Comp. Neurol. **273**:207–223.
113. **Oesch, S., L. Degen, and C. Beglinger.** 2005. Effect of a protein preload on food intake and satiety feelings in response to duodenal fat perfusions in healthy male subjects. Am J Physiol Regul Integr Comp Physiol **289**:R1042-7.
114. **Oesch, S., C. Ruegg, B. Fischer, L. Degen, and C. Beglinger.** 2006. Effect of gastric distension prior to eating on food intake and feelings of satiety in humans. Physiol Behav **87**:903–910.
115. **Pain, V. M.** 1994. Translational control during amino acid starvation. Biochimie **76**:718–728.
116. **Pérez, C., K. Ackroff, and A. Sclafani.** 1996. Carbohydrate- and protein-conditioned flavor preferences: Effects of nutrient preloads. Physiology & Behavior **59**:467–474.
117. **Perez, C., F. Lucas, and A. Sclafani.** 1995. Carbohydrate, fat, and protein condition similar flavor preferences in rats using an oral-delay procedure. Physiol Behav **57**:549–554.
118. **Peters, J. C., and A. E. Harper.** 1987. Acute effects of dietary protein on food intake, tissue amino acids, and brain serotonin. Am J Physiol **252**:R902-14.
119. **Phositlimpagul, A., G. L. Edwards, and M. J. Azain.** 1. Hepatic vagotomy disrupts somatotropin-induced protein selection. Physiology & Behavior **75**:193–200.
120. **Pi, M., L. Chen, M.-Z. Huang, W. Zhu, B. Ringhofer, J. Luo, L. Christenson, B. Li, J. Zhang, P. D. Jackson, P. Faber, K. R. Brunden, J. J. Harrington, and L. D. Quarles.** 2008. GPRC6A null mice exhibit osteopenia, feminization and metabolic syndrome. PLoS One **3**:e3858.
121. **Pichon, L., M. Potier, D. Tome, T. Mikogami, B. Laplaize, C. Martin-Rouas, and G. Fromentin.** 2008. High-protein diets containing different milk protein fractions differently influence energy intake and adiposity in the rat. Br. J. Nutr. **99**:739–748.

122. **Purpera, M. N., L. Shen, M. Taghavi, H. Munzberg, R. J. Martin, S. M. Hutson, and C. D. Morrison.** 2012. Impaired branched chain amino acid metabolism alters feeding behavior and increases orexigenic neuropeptide expression in the hypothalamus. J Endocrinol **212**:85–94.
123. **Raben, A., L. Agerholm-Larsen, A. Flint, J. J. Holst, and A. Astrup.** 2003. Meals with similar energy densities but rich in protein, fat, carbohydrate, or alcohol have different effects on energy expenditure and substrate metabolism but not on appetite and energy intake. Am J Clin Nutr **77**:91–100.
124. **Raubenheimer, D., and S. J. Simpson.** 1997. Integrative models of nutrient balancing: application to insects and vertebrates. Nutr Res Rev **10**:151–179.
125. **Raybould, H. E.** 1991. Capsaicin-sensitive vagal afferents and CCK in inhibition of gastric motor function induced by intestinal nutrients. Peptides **12**:1279–1283.
126. **Reidelberger, R. D., L. Kelsey, D. Heimann, and M. Hulce.** 2003. Effects of peripheral CCK receptor blockade on gastric emptying in rats. Am J Physiol Regul Integr Comp Physiol **284**:R66-75.
127. **Reimann, F., M. Maziarz, G. Flock, A. M. Habib, D. J. Drucker, and F. M. Gribble.** 2005. Characterization and functional role of voltage gated cation conductances in the glucagon-like peptide-1 secreting GLUTag cell line. The Journal of Physiology **563**:161–175.
128. **Reimann, F., G. Tolhurst, and F. M. Gribble.** 2012. G-Protein-Coupled Receptors in Intestinal Chemosensation. Cell Metabolism **15**:421–431.
129. **Reimann, F., P. S. Ward, and F. M. Gribble.** 2006. Signaling Mechanisms Underlying the Release of Glucagon-Like Peptide 1. Diabetes **55**:S78-S85.
130. **Roberts, T. J., M. J. Azain, B. D. White, and R. J. Martin.** 1995. Rats treated with somatotropin select diets higher in protein. The Journal of Nutrition **125**:2669–2678.
131. **Russell, M. C., T. J. Koehnle, J. A. Barrett, J. E. Blevins, and D. W. Gietzen.** 2003. The rapid anorectic response to a threonine imbalanced diet is decreased by injection of threonine into the anterior piriform cortex of rats. Nutr Neurosci **6**:247–251.
132. **Saidak, Z., M. Brazier, S. Kamel, and R. Mentaverri.** 2009. Agonists and Allosteric Modulators of the Calcium-Sensing Receptor and Their Therapeutic Applications. Molecular Pharmacology **76**:1131–1144.
133. **Schmelzle, T., and M. N. Hall.** 2000. TOR, a Central Controller of Cell Growth. Cell **103**:253–262.
134. **Schonhoff, S., L. Baggio, C. Ratineau, S. K. Ray, J. Lindner, M. A. Magnuson, D. J. Drucker, and A. B. Leiter.** 2005. Energy homeostasis and gastrointestinal endocrine differentiation do not require the anorectic hormone peptide YY. Mol Cell Biol **25**:4189–4199.
135. **Schubert, M. L.** 2003. Gastric secretion. Curr Opin Gastroenterol **19**:519–525.
136. **Schubert, M. L., and D. A. Peura.** 2008. Control of Gastric Acid Secretion in Health and Disease. Gastroenterology **134**:1842–1860.
137. **Schwartz, G. J.** 2006. Integrative capacity of the caudal brainstem in the control of food intake. Philosophical Transactions of the Royal Society B: Biological Sciences **361**:1275–1280.
138. **Shin, A. C., H. Zheng, R. L. Townsend, D. L. Sigalet, and H.-R. Berthoud.** 2010. Meal-induced hormone responses in a rat model of Roux-en-Y gastric bypass surgery. Endocrinology **151**:1588–1597.
139. **Simpson, S. J., and D. Raubenheimer.** 1999. Assuaging nutritional complexity: a geometrical approach. Proc Nutr Soc **58**:779–789.
140. **Simpson, S. J., and D. Raubenheimer.** 2005. Obesity: the protein leverage hypothesis. Obes Rev **6**:133–142.
141. **Smeets, A. J., S. Soenen, N. D. Luscombe-Marsh, O. Ueland, and M. S. Westerterp-Plantenga.** 2008. Energy expenditure, satiety, and plasma ghrelin, glucagon-like peptide 1, and peptide tyrosine-tyrosine concentrations following a single high-protein lunch. J Nutr **138**:698–702.
142. **Sorensen, A., D. Mayntz, D. Raubenheimer, and S. J. Simpson.** 2008. Protein-leverage in mice: the geometry of macronutrient balancing and consequences for fat deposition. Obesity (Silver Spring) **16**:566–571.

143. **Stephens, J. R., R. F. Woolson, and A. R. Cooke.** 1975. Effects of essential and nonessential amino acids on gastric emptying in the dog. Gastroenterology **69**:920-927.
144. **Szarka, L. A., and M. Camilleri.** 2009. Methods for measurement of gastric motility. American Journal of Physiology - Gastrointestinal and Liver Physiology **296**:G461-G475.
145. **Tanaka, K., S. Inoue, H. Nagase, Y. Takamura, and A. Niijima.** 1990. Amino acid sensors sensitive to alanine and leucine exist in the hepato-portal system in the rat. J Auton Nerv Syst **31**:41–46.
146. **Timmons, J. A., K. Baar, P. K. Davidsen, and P. J. Atherton.** 2012. Is irisin a human exercise gene? Nature **488**:E9.
147. **Tolhurst, G., Y. Zheng, H. E. Parker, A. M. Habib, F. Reimann, and F. M. Gribble.** 2011. Glutamine triggers and potentiates glucagon-like peptide-1 secretion by raising cytosolic Ca2+ and cAMP. Endocrinology **152**:405–413.
148. **Tome, D., J. Schwarz, N. Darcel, and G. Fromentin.** 2009. Protein, amino acids, vagus nerve signaling, and the brain. Am J Clin Nutr **90**:838S-843S.
149. **Tome, D., J. Schwarz, N. Darcel, and G. Fromentin.** 2009. Protein, amino acids, vagus nerve signaling, and the brain. Am J Clin Nutr **90**:838S-843S.
150. **Torii, K., and A. Niijima.** 2001. Effect of lysine on afferent activity of the hepatic branch of the vagus nerve in normal and L-lysine-deficient rats. Physiol Behav **72**:685–690.
151. **Torii, K., and A. Niijima.** 2001. Effect of lysine on afferent activity of the hepatic branch of the vagus nerve in normal and L-lysine-deficient rats. Physiol Behav **72**:685–690.
152. **Tsubuku, S., M. Mochizuki, K. Mawatari, M. Smriga, and T. Kimura.** 2004. Thirteen-week oral toxicity study of L-lysine hydrochloride in rats. Int J Toxicol **23**:113–118.
153. **Tsurugizawa, T., A. Uematsu, E. Nakamura, M. Hasumura, M. Hirota, T. Kondoh, H. Uneyama, and K. Torii.** 2009. Mechanisms of neural response to gastrointestinal nutritive stimuli: the gut-brain axis. Gastroenterology **137**:262–273.
154. **Uneyama, H., A. Niijima, A. San Gabriel, and K. Torii.** 2006. Luminal amino acid sensing in the rat gastric mucosa. Am J Physiol Gastrointest Liver Physiol **291**:G1163-70.
155. **Verrey, F., D. Singer, T. Ramadan, R. N. Vuille-dit-Bille, L. Mariotta, and S. M. R. Camargo.** 2009. Kidney amino acid transport. Pflugers Arch **458**:53–60.
156. **Vilanayur Ramachandran**. Encyclopedia of Human Behavior. Second Edition.
157. **Walter F. Boron, and Emile L. Boulpaep**. Medical physiology. second edition.
158. **Wang, G.-J., D. Tomasi, W. Backus, R. Wang, F. Telang, A. Geliebter, J. Korner, A. Bauman, J. S. Fowler, P. K. Thanos, and N. D. Volkow.** 2008. Gastric distention activates satiety circuitry in the human brain. NeuroImage **39**:1824–1831.
159. **Wang, Y., R. Chandra, L. A. Samsa, B. Gooch, B. E. Fee, J. M. Cook, S. R. Vigna, A. O. Grant, and R. A. Liddle.** 2010. Amino Acids Stimulate Cholecystokinin Release Through the Calcium-Sensing Receptor. American Journal of Physiology - Gastrointestinal and Liver Physiology.
160. **Watts, A. G., A. M. Khan, G. Sanchez-Watts, D. Salter, and C. M. Neuner.** 2006. Activation in neural networks controlling ingestive behaviors: what does it mean, and how do we map and measure it? Physiol Behav **89**:501–510.
161. **Wellendorph, P., and H. Bräuner-Osborne.** 2004. Molecular cloning, expression, and sequence analysis of GPRC6A, a novel family C G-protein-coupled receptor. Gene **335**:37–46.
162. **Wellendorph, P., K. B. Hansen, A. Balsgaard, J. R. Greenwood, J. Egebjerg, and H. Bräuner-Osborne.** 2005. Deorphanization of GPRC6A: A Promiscuous l-α-Amino Acid Receptor with Preference for Basic Amino Acids. Molecular Pharmacology **67**:589–597.
163. **Wettschureck, N., E. Lee, S. K. Libutti, S. Offermanns, P. G. Robey, and A. M. Spiegel.** 2007. Parathyroid-Specific Double Knockout of Gq and G11 α-Subunits Leads to a Phenotype Resembling Germline Knockout of the Extracellular Ca2+-Sensing Receptor. Molecular Endocrinology **21**:274–280.
164. **White, B. D., B. He, R. G. Dean, and R. J. Martin.** 1994. Low protein diets increase neuropeptide Y gene expression in the basomedial hypothalamus of rats. J Nutr **124**:1152–1160.

165. **White, B. D., M. H. Porter, and R. J. Martin.** 2000. Effects of age on the feeding response to moderately low dietary protein in rats. Physiol Behav **68**:673–681.
166. **Whitedouble dagger, B. D., M. H. Porter, and R. J. Martin.** 2000. Protein selection, food intake, and body composition in response to the amount of dietary protein. Physiol Behav **69**:383–389.
167. **Woods, S. C.** 2009. The control of food intake: behavioral versus molecular perspectives. Cell Metab **9**:489–498.
168. **Woods, S. C., and W. Langhans.** 2012. Inconsistencies in the assessment of food intake. Am J Physiol Endocrinol Metab **303**:E1408-18.
169. **Wu, G.** 2009. Amino acids: metabolism, functions, and nutrition. Amino Acids **37**:1–17.
170. **Young, S. H., O. Rey, C. Sternini, and E. Rozengurt.** 2010. Amino acid sensing by enteroendocrine STC-1 cells: role of the Na+-coupled neutral amino acid transporter 2. Am J Physiol Cell Physiol **298**:C1401-13.
171. **Zhang, Y., K. Guo, R. E. LeBlanc, D. Loh, G. J. Schwartz, and Y.-H. Yu.** 2007. Increasing dietary leucine intake reduces diet-induced obesity and improves glucose and cholesterol metabolism in mice via multimechanisms. Diabetes **56**:1647–1654.
172. **Zhao, G. Q., Y. Zhang, M. A. Hoon, J. Chandrashekar, I. Erlenbach, N. J. P. Ryba, and C. S. Zuker.** 2003. The receptors for mammalian sweet and umami taste. Cell **115**:255–266.

10. Curriculum Vitae

Josua Jordi

Personal	Nationality: Swiss	Birthday: 28.9.1985
	Place of Birth: Zurich, CH	Civil Status: single

Qualification Grown up & basic education in the Philippines, Portugal, Argentina & Switzerland, swiss army officer, bachelor & master of science in biochemistry ETHZ, present PhD student in integrative molecular medicine UZH

University	2010 – present	PhD in integrative molecular medicine, laboratory of Prof. Dr. F. Verrey & Prof. Dr. T. Lutz, University of Zurich (UZH) Title: "Individual Amino Acids – Hungry Brain and Mobile Gut"
	2008 – 2010	Master of science in biochemistry, laboratory of Prof. Dr. U. Sauer, Swiss Federal Institute of Technology Zurich (ETHZ) Title: "The Phases of Life in *Saccharomyces Cerevisiae*" summa cum laude
	2005 – 2008	Bachelor of science in biology with chemical focus ETHZ
Award	2012	Oetliker prize (2'600sFR), young investigator award of the Swiss physiological society
	2012	Grant for "Vision 2020" lecture series (70'000sFR), UZH
	2012	Travel award (1'400sFR), Swiss physiological society
	2009	Winner of the european novartis biotechnology leadership camp as a team and an individual
	2009	Best grant, master course ETHZ
Committee	2010 – 2013	Commission of the PhD-program in integrative molecular medicine (student representative), UZH
	2010 – 2012	Career possibilities seminar committee, UZH
	2010	Master celebration ceremony committee, ETHZ
Teaching	2010 – 2011	Physiology practicum for first & second year medical students UZH (ca. 100h, practical assistance)
	2010 – 2011	Physiology practicum for third year biology students UZH (ca. 100h, supervision, teaching & grading)
	2011	Supervision of a bachelor student research project

Military	2012 – present	ABC Laboratory Spiez, Lab B Officer
	2005 – 2012	Mountain infantry officer, Geb Inf 17/2 & 17/4
	2004 – 2005	Training as infantry officer (second lieutenant)
Education	2001 – 2004	Gymnasium Thun – Schadau (economics & law), Switzerland
	1997 – 2001	Goethe Schule Buenos Aires, Argentina
	1993 – 1997	Deutsche Schule in Estoril & Lissabon, Portugal
	1991 – 1993	Eurocampus Manila, Philippines

Language German (mother tongue), English (fluent), Spanish & French (good knowledge), Portuguese & Italian (knowledge)

Leisure Friends, badminton (4^{th} & 3^{rd} league), endurance sports (Ironman, SOLA, Strongmen), windsurfing, beach volleyball, snowboarding

Conference	2013	Zurich center of integrative human physiology symposium (poster), Zurich
	2013	Experimental biology meeting (poster), Boston
	2012	Swiss innovation forum, Basel
	2012	Meeting of the Swiss physiological society (talk), Fribourg
	2012	Meeting of the society for the study of ingestive behaviour (poster), Zurich
	2012	Swiss economic forum, Interlaken
	2012	Experimental biology meeting (poster), San Diego
	2011	Zurich center of integrative human physiology symposium (talk), Zurich

Method

PhD	Rat model - basics (FELASA Category B), behavioural studies, surgery (area postrema lesion, capsaicin, hepatic portal vain catheter), RT-PCR, IHC, mCT *in vivo* imaging, radioactive and dye tracing studies
Master	Yeast model - basics, 4D-quantitative microscopy, metabolomics (HPLC, GC-TOF, LC-MS/MS)

11. Acknowledgments

This is a tribute to all the people who have helped me during my doctoral studies and/or created an enjoyable and exciting atmosphere in the lab and in the office. Thank you!

Especially I want to thank Thomas and François, who both supervised and scientifically guided me in the last three years. They always were open to new approaches and ideas, gave critically suggestion and comments, and supported my desire for independence early on. I particularly appreciated that they accepted the challenge to work with a “yeast boy” in this interdisciplinary collaborative project. Due to my shared PhD, I had the change to interact with many scientists from different backgrounds, got insights into different labs and learned many new things about related fields. Most enjoyable were to me the interesting diner conversations which we could share in Zurich and Boston. Thank you very much for all your support!

I would like to acknowledge my committee members Daniel and Carsten for their contribution to the success of this thesis and giving valuable input whenever needed. We also appreciated Daniel’s coming always from Paris! Merci!

Huge thanks goes to Brigitte for the great and endless support during experiments, for the stimulating discussion about science and the world, for the cheer-up after the rejected FK, for the great army packages and for everything else. Thank you very, very much!

A particularly other important person for my studies was Simone – Thank you very much for giving me such a great start at the Institute of Physiology, for sharing the passion and fascination of small and bigger results, for tuning my talks to the very best and for all your motivation and energy. Thank you so much!

I also want to thank Christina for smoothing the waves which I might have generated at Tierspital, for the battles through animal scheduling, for the first insights into behavior and neuroscience studies and for supporting the project wherever possible. Thank you!

Many thanks go to “the human part” of our cooperative group, Andreas, Carolin, Werner, Sena, Oliver and Raphael for inspiring, constructive, intense and funny meetings at the hospital or here at Irchel, for the “interesting” gastrointestinal effects during self-testing and for the wonderful discussions about linearity, exponential and other functions. Thank you!

I greatly appreciated my time at the imMED commission therefore I want to thank Heidi, Thierry, Christian, Thomas, Carsten and Dominick for giving me this opportunity and their trust!

Naturally I want to thank also my two “science”-families – all the current and former Verrey and Lutz lab members. Thank you for all the fun we had together in- and outside the lab, for joining with so much enthusiasm at the SOLA race and at different parties, for sharing positive and negative emotions, for helping out here and there, and for teaching new and sometimes crazy things. Particularly I want to thank Adriano for the super trips in the US and all the positive things we could share and discussion from scientific fascinations to fundamental future decisions. The same holds for Lorenz, my best science buddy from Zurich, thank you for the great time in the lab, bars and on the bike! Thank you guys and I wish you all the best in your future!

Christa for all the support, encouragement and love I could wish for! Merci!

Mama, Papa and Andrea for their endless support, motivation, energy since the very beginning! Danke!